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P. 611 B

ALYTES

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The genus *Chiasmocleis* Méhely, 1904 (Anura, Microhylidae) in the Atlantic Rain Forest of Brazil, with description of three new species

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A taxonomic study of the species of the genus *Chiasmocleis* occurring in the Atlantic Rain Forest of eastern Brazil is presented. *Chiasmocleis bicegoi* Miranda-Ribeiro, 1920 is synonymized with *C. albopunctata* (Boettger, 1885), and rendered extralimital for the present work. The synonymization of *C. urbanae* Bokermann, 1952 with *C. leucosticta* (Boulenger, 1888) is reaffirmed. *Chiasmocleis leucosticta* and *C. schubarti* Bokermann, 1952 are redescribed, and three new species are described.

INTRODUCTION

The genus *Chiasmocleis*, proposed by MÉHELY (1904), currently contains 12 species, distributed from Panama to southern South America, north and east of the Andes (FROST, 1985). The validity of the species included in this genus has not been assessed by a modern review. Materials from the Atlantic Forests of Brazil indicate such an assessment is needed. Herein, we present a review of the species of *Chiasmocleis* inhabiting the Atlantic Rain Forest of eastern Brazil. This systematic review includes: (1) re-examination of previously described taxa; (2) redescription of the valid species; and (3) description of three new species.

MATERIALS AND METHODS

Abbreviations of the collections housing specimens are: BMNH (Natural History Museum, London, United Kingdom); CFBH (Célio F. B. HADDAD Collection, Universidade

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Estadual Paulista, Rio Claro, SP, Brazil); EI (Eugênio IZECKSOHN Collection, Universidade Federal Rural do Rio de Janeiro, RJ, Brazil); MNRJ (Museu Nacional do Rio de Janeiro, RJ, Brazil); MZUSP (Museu de Zoologia, Universidade de São Paulo, SP, Brazil); SPCS (Sérgio POTSCH DE CARVALHO E SILVA Collection, Universidade Federal do Rio de Janeiro, RJ, Brazil); UFV (Museu de História Natural, Universidade Federal de Viçosa, MG, Brazil). Specimens examined are listed in app. 1.

Measurements, in millimeters, are: SVL (snout-vent length); HL (head length); HW (head width); IND (internarial distance); END (eye to nostril distance); ED (eye diameter); UEW (upper eyelid width); IOD (interorbital distance); THL (thigh length); TL (tibia length); FL (foot length).

NOMENCLATURAL HISTORY

Each of the five names proposed for a member of the genus *Chiasmocleis* occurring in the Atlantic Rain Forest is discussed in chronological order.

ENGYSTOMA LEUCOSTICTUM BOULENGER, 1888

This species was described on the basis of a female specimen (type not stated; BMNH 88.4.23.3 registered as holotype, according to FROST, 1985) collected at "Sierra do Catharina, Province Santa Catharina, Brazil" (BOULENGER, 1888). Currently this locality is in the Catarina Mountain Range, in the state of Santa Catarina, southern Brazil. PARKER (1934) transferred the species to the genus *Chiasmocleis*, under the combination *Chiasmocleis leucosticta*.

CHIASMOCLEIS BICEGOI MIRANDA-RIBEIRO, 1920

The species was described based on a single specimen from "Os Perus, S. Paulo" (MIRANDA-RIBEIRO, 1920). Currently, this locality is a suburb within the western side of the city of São Paulo, state of São Paulo, Brazil. The holotype (MZUSP 924, formerly MZUSP 595), a juvenile (15.0 mm SVL) of undetermined sex, is in very poor condition, totally discoloured, the ventral and pectoral regions dilacerated, and arms and legs almost totally destroyed. Direct comparison of the holotype of *C. bicegoi* with a recently collected young specimen of *Chiasmocleis albopunctata* Boettger, 1885, obtained at Iperó, state of São Paulo (MNRJ 17324, 13.9 mm SVL), indicates that the former is a junior synonym of the latter. *Chiasmocleis albopunctata* is an open area inhabitant, occurring in the "cerrados" of Brazil in the states of São Paulo, Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul, and Distrito Federal, and in Paraguay and Bolivia. Iperó is about 90 km from the type locality of *C. bicegoi*, and it is perfectly acceptable that the species occurred near the city of São Paulo at the end of the last century, when the holotype was collected by BICEGO. The shape of the snout, although with some deformation in the holotype of *C. bicegoi*, is coincident in both specimens; vestiges of the white blotches on the snout and dorsolateral region of the body

Source : MNHN, Paris

characteristic of *C. albopunctata* are still observed in the holotype of *C. bicegoi*. Thus, synonymization of *C. bicegoi* with *C. albopunctata* renders it extralimital for the present work. The citation of *C. bicegoi* for Itaguaí, state of Rio de Janeiro, by BOGART & NELSON (1976) applies to a new species described in the account below.

NECTODACTYLUS SPINULOSUS MIRANDA-RIBEIRO, 1924

The genus *Nectodactylus* was proposed by MIRANDA-RIBEIRO (1924) to accomodate a new species, named *N. spinulosus* (syntypes MNRJ 525 and 5582), from "Humboldt – Sta. Catharina – Brasilia" (currently Corupá, state of Santa Catarina, Brazil). The genus was synonymized with *Chiasmocleis* by PARKER (1934), resulting in the combination *Chiasmocleis spinulosa*. Later, CARVALHO (1954) synonymized *Nectodactylus spinulosus* with *Engystoma leucostictum*, thus recognizing *Chiasmocleis leucosticta* as the valid name for the species.

CHIASMOCLEIS SCHUBARTI BOKERMANN, 1952

The species was described based on a young specimen (holotype MZUSP 2309) from "Córrego Juncado, Linhares", state of Espírito Santo, Brazil (BOKERMANN, 1952). This is a perfectly valid and recognizable species.

CHIASMOCLEIS URBANAE BOKERMANN, 1952

The species was described on the basis of a purported male specimen (holotype MZUSP 9033) from "Ilha de São Sebastião", on the coast of the state of São Paulo, Brazil (BOKERMANN, 1952). *Chiasmocleis urbanae* was synonymized with *C. leucosticta* by BOKERMANN (1966); this action was not recognized in FROST (1985), where *C. urbanae* was treated as a valid species. Examination of the holotype of *C. urbanae* showed it to be a female specimen and identical to specimens of *C. leucosticta* from the state of Santa Catarina and the southern portion of the state of São Paulo. Consequently, we agree with BOKERMANN (1966) and consider *C. urbanae* to be a synonym of *C. leucosticta*.

SPECIES ACCOUNTS

Chiasmocleis leucosticta (Boulenger, 1888)

Engystoma leucostictum Boulenger, 1888.
Chiasmocleis leucosticta: PARKER, 1934.
Nectodactylus spinulosus Miranda-Ribeiro, 1924.
Chiasmocleis spinulosa: PARKER, 1934.
Chiasmocleis urbanae Bokermann, 1952.

Diagnosis. – A medium sized species of *Chiasmocleis* diagnosed by the following combination of characters: (1) SVL 19.0-22.8 mm in males, 21.8-25.5 mm in females; (2) body trunk ovoid;

Source : MNHN, Paris

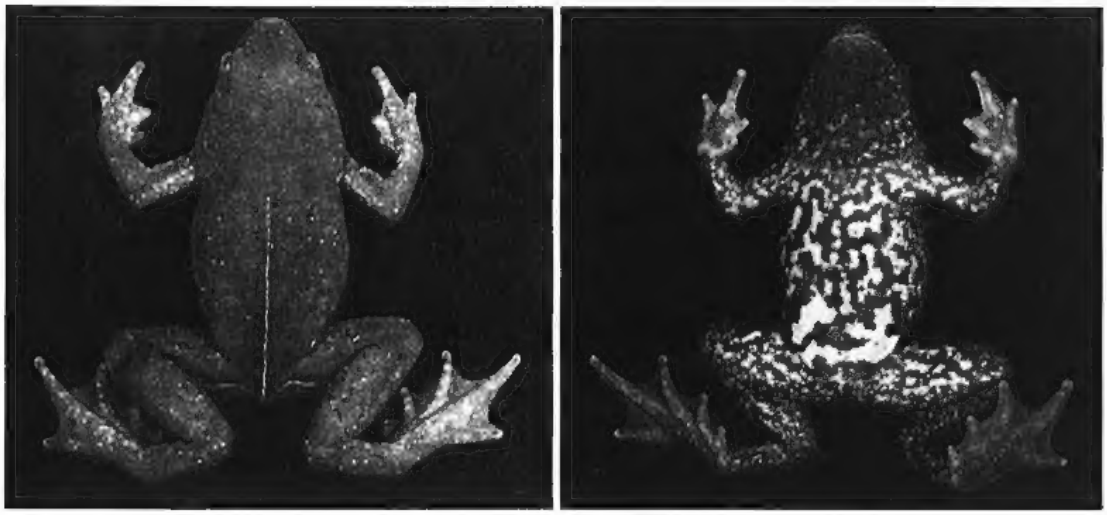

Fig. 1. – Dorsal and ventral views of *Chiasmocleis leucosticta* (Boulenger, 1888) (MNRJ 17901).

(3) snout short, tip slightly truncate in dorsal view, and slightly protruding in lateral profile; (4) hand and foot of male webbed, of female half webbed; (5) fingers and toes lacking disks, fringed on free parts in females; (6) fingers and toes with few, small lateral dermal spines only in males; (7) dorsal surfaces with small dermal spines; (8) in preservative, color on dorsum dark brown with small white dots irregularly distributed, and a light longitudinal mid-dorsal line; (9) posterior sides of legs with a light longitudinal line; (10) venter roughly marbled in dark brown and pale cream.

Description. – Size medium for the genus; body trunk ovoid (fig. 1); head short, as broad as long; nostrils at the tip of snout, not protuberant, and directed laterally; snout short, tip slightly truncate in dorsal view (fig. 2 A), and slightly protruding in lateral profile (fig. 2 B); internarial distance smaller than eye to nostril distance, and only slightly smaller than eye diameter; eye diameter less than eye to nostril distance; canthus rostralis only slightly defined; loreal region oblique, flat; lips not flared; eyes small, only slightly protruding; upper eyelid width one third of the interorbital space; interorbital area flat; cranial crests and occipital fold absent; postorbital fold present; tympanum absent; upper jaw projecting beyond lower; mandible with truncate, trilobed anterior margin; tongue large, ovoid; choanae small, rounded, widely separated; a small, subgular vocal sac present.

Arms slender, lacking tubercles and crests on forearm. Hand of male (fig. 2 C) webbed, of female (fig. 3 A) half webbed; fingers lacking disks, fringed on free parts in females, with lateral dermal spines only in males; finger length I < II < IV < III; subarticular tubercles well developed, rounded; supernumerary tubercles absent; palmar tubercle large, divided in two parts; thenar tubercle large, rounded, at the base of finger I.

Legs short, robust; knee and heel lacking tubercles; tibial and tarsal ridges absent. Foot of male (fig. 2 D) webbed, of female (fig. 3 B) half webbed; toes lacking disks and fringed on free parts; toe length I < II < V < III < IV; toes with few, small lateral dermal spines in males,

Source : MNHN, Paris

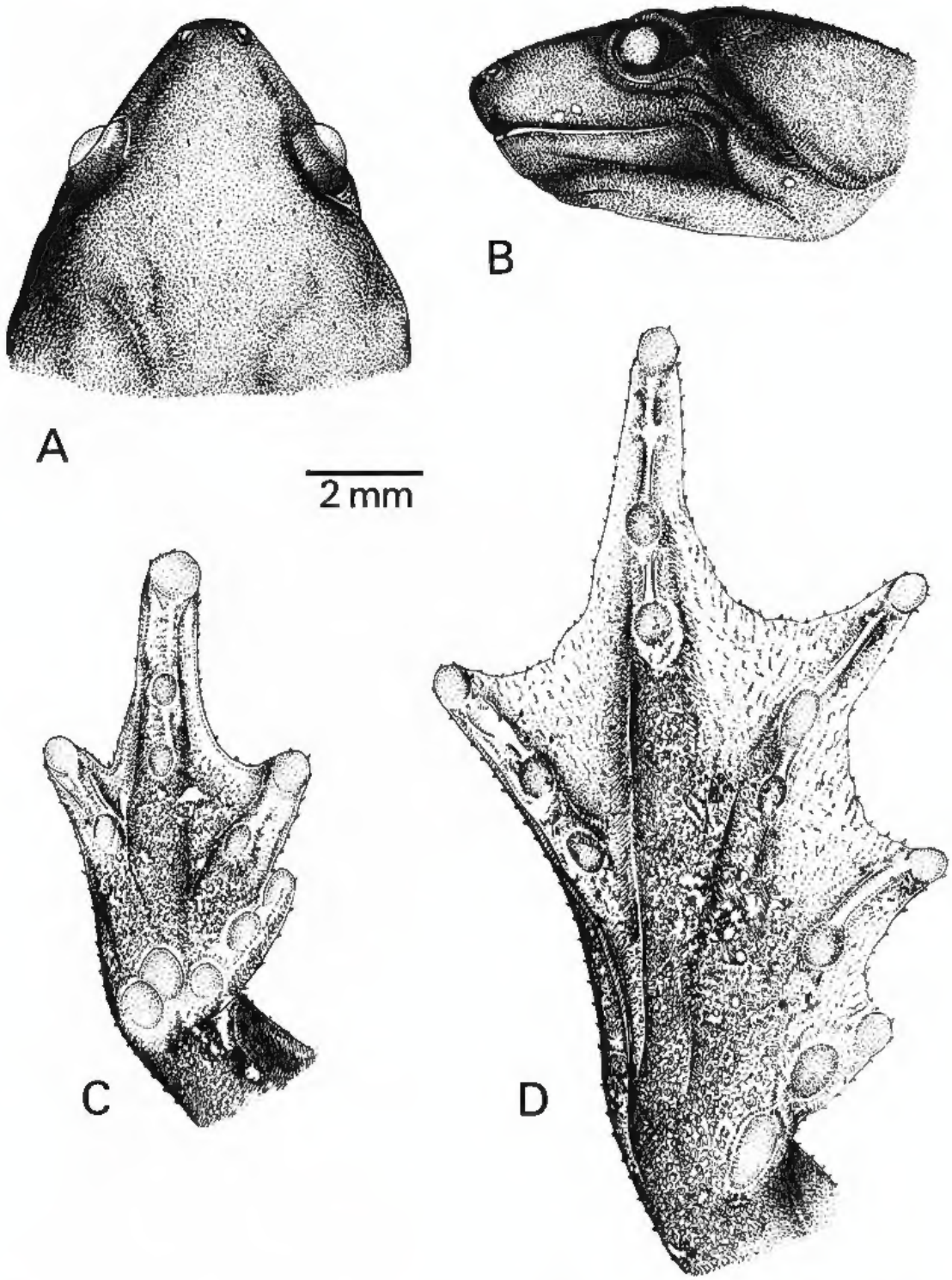

Fig. 2. – *Chiasmocleis leucosticta* (Boulenger, 1888), male (MNRJ 17901). Dorsal (A) and ventral (B) views of head. Hand (C). Foot (D).

Source : MNHN, Paris

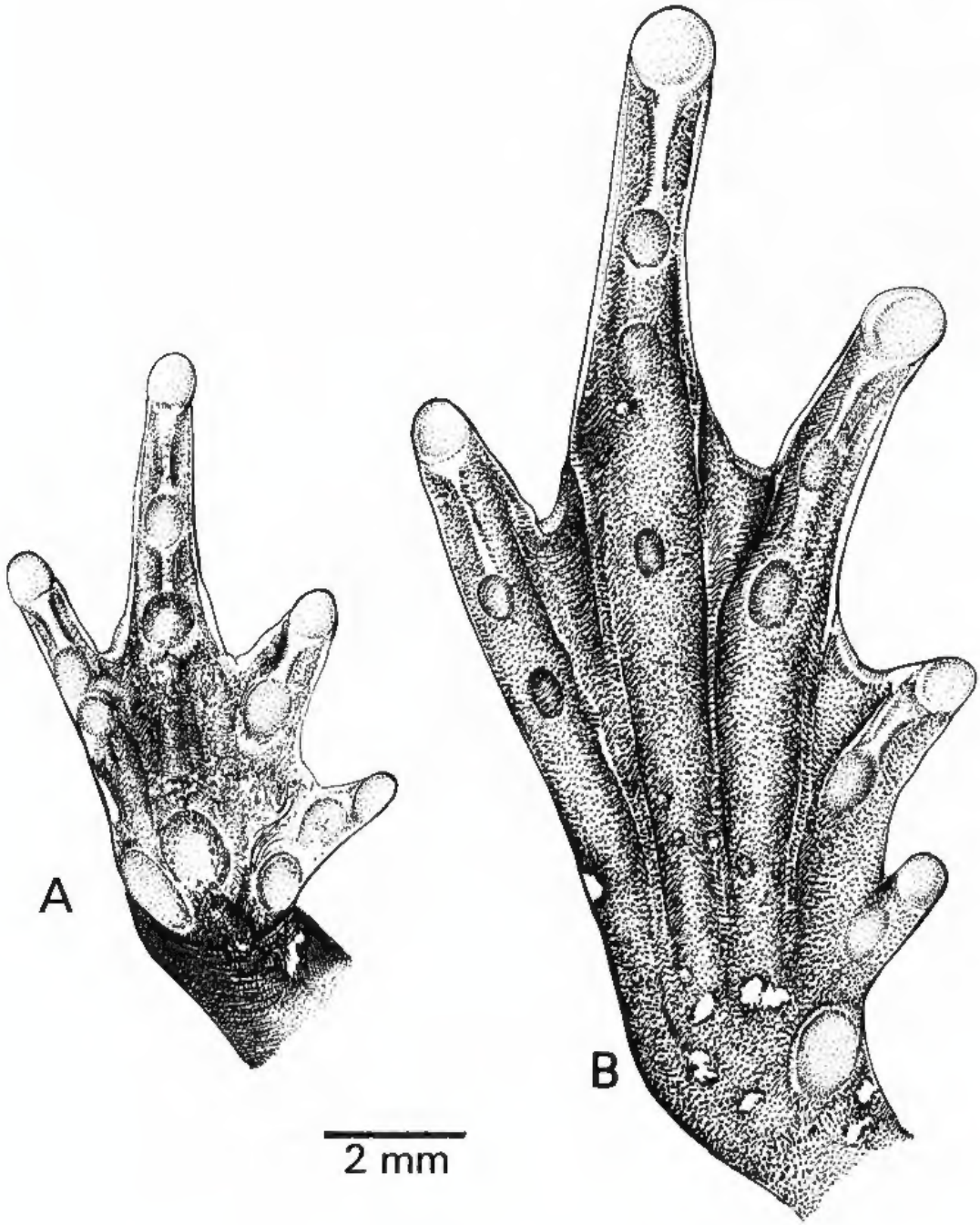

Fig. 3. – *Chiasmocleis leucosticta* (Boulenger, 1888), female (MNRJ 17902). Hand (A). Foot (B).

Source : MNHN, Paris

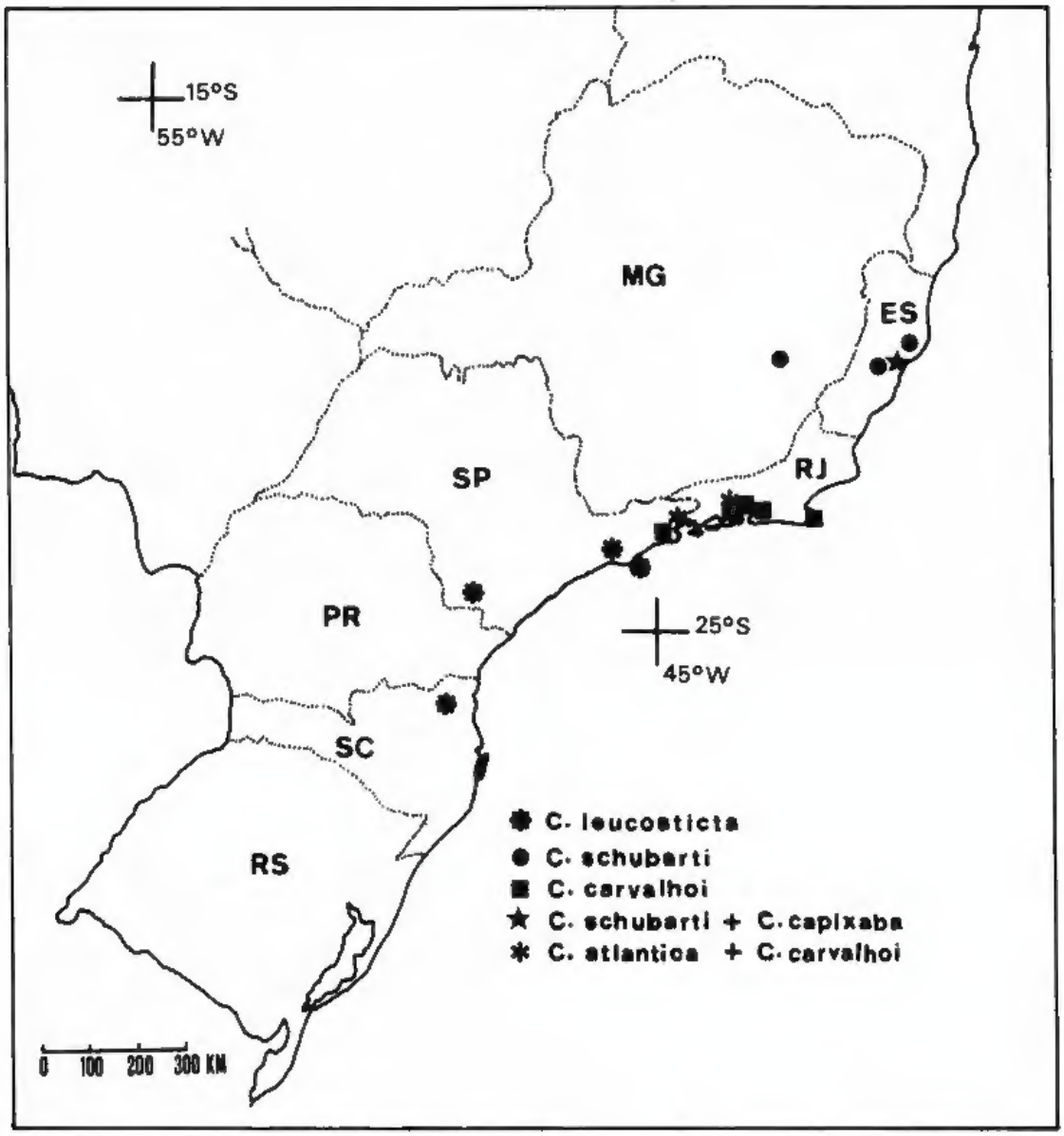

Fig. 4. – Geographical distribution of the species of *Chiasmocleis* in the Atlantic Rain Forest of Brazil (states of southeastern and southern regions of Brazil: MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo; PR; Paraná; SC, Santa Catarina; RS, Rio Grande do Sul).

lacking in females; subarticular tubercles well developed, rounded; supernumerary tubercles absent; an oval inner, but no outer, metatarsal tubercle. Thigh length slightly less than tibia length; knee and elbow widely separated with limbs adpressed to sides of body; combined thigh and tibia length approximately 83 % of snout-vent length in males, 85 % in females; heels slightly superposed when flexed legs held at right angles to body; foot length approximately 66 % of snout-vent length in males, 69 % in females.

Source : MNHN, Paris

Skin smooth above and beneath; dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines, more numerous in males. Anal opening not modified, lacking para-anal tubercles and glands around anus.

In preservative (70 % ETOH), color on dorsum dark brown with small white dots irregularly distributed; a light longitudinal mid-dorsal line meeting, above the anus, another light longitudinal line present on the posterior sides of legs. Venter roughly marbled in dark brown and pale cream; male throat infuscated.

Variation. – Variation in measurements is presented in tab. 1. Females are larger than males, with interdigital webbing less developed. In some females, a light line is present on ventral surface of arms and pectoral region, where it assumes a V-shape, crossed by a perpendicular light longitudinal mid-ventral line.

Geographical distribution. – Known from the states of São Paulo and Santa Catarina, in southeastern and southern Brazil (fig. 4). The occurrence of the species in the state of Paraná is predictable.

Remarks. – Specimens of *C. leucosticta* were collected in temporary ponds inside the forest, from about 30 to 800 m above sea level. The reproduction is explosive, when numerous male and female specimens congregate at the reproductive sites. Kasahara & Haddad (1997) reported a population of *C. leucosticta* from Ribeirão Branco, state of São Paulo, to be tetraploid (4 n = 48).

Chiasmocleis schubarti Bokermann, 1952

Chiasmocleis schubarti Bokermann, 1952.

Diagnosis. – A medium sized species of *Chiasmocleis* diagnosed by the following combination of characters: (1) SVL 19.2-26.3 mm in males, 20.4-34.5 mm in females; (2) body trunk ovoid; (3) snout short, tip rounded in dorsal and lateral profile; (4) hand not webbed, foot only slightly webbed at the base of toes in both sexes; (5) fingers and toes lacking disks, slightly fringed; (6) fingers and toes of males with small, lateral dermal spines, absent in females; (7) dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region; (8) in preservative, color on dorsum uniformly dark brown; (9) posterior sides of thighs with a light longitudinal line; (10) venter roughly marbled in dark brown and pale cream.

Description. – Size medium for the genus; body trunk ovoid (fig. 5); head short, broader than long; nostrils near the tip of snout, not protuberant, and directed laterally; snout short, tip rounded in dorsal and lateral profile (fig. 6 A-B); internarial distance smaller than eye to nostril distance, and slightly larger than eye diameter; eye diameter less than eye to nostril distance; canthus rostralis only slightly defined; loreal region oblique, flat; lips not flared; eyes small, only slightly protruding; upper eyelid width one third of the interorbital space; interorbital area flat; cranial crests and occipital fold absent; postorbital fold present; tympanum absent; upper jaw projecting beyond lower; mandible with truncate, trilobed anterior margin; tongue large, ovoid; choanae small, rounded, widely separated; a small, subgular vocal sac present.

Source : MNHN, Paris

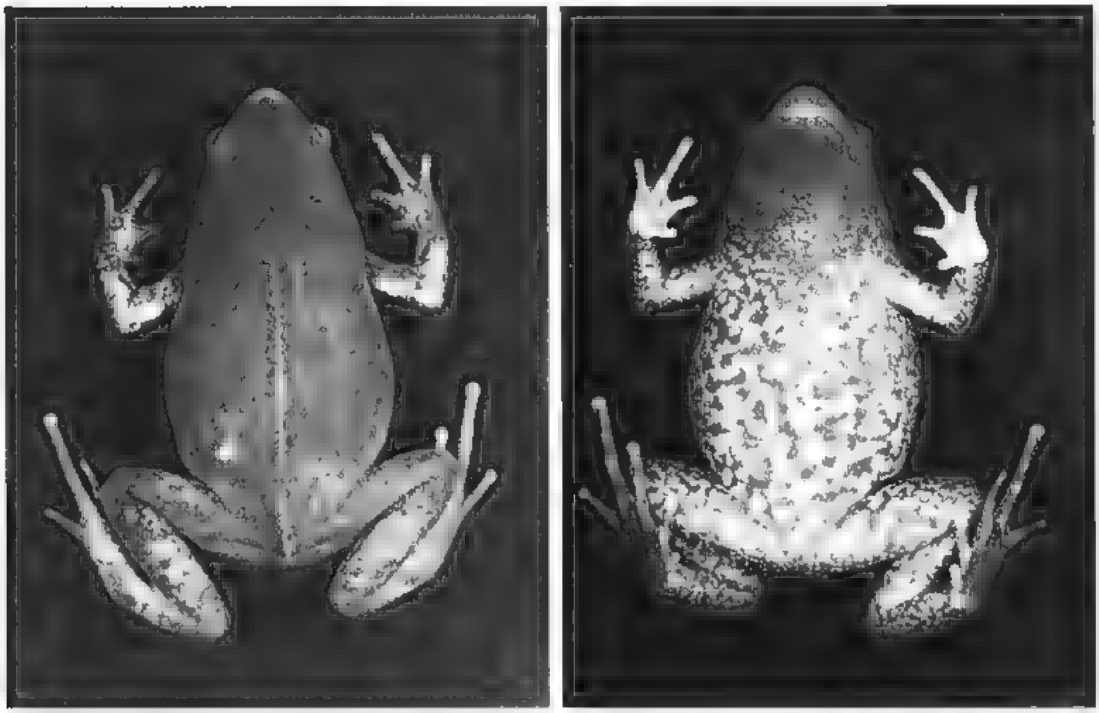

Fig. 5 – Dorsal and ventral views of *Chiasmocleis schubarti* Bokermann, 1952 (MNRJ 17538).

Arms slender, lacking tubercles and crests on forearm. Hand (fig. 6 C) not webbed in both sexes; fingers lacking disks, slightly fringed; fingers with small, lateral dermal spines in males, absent in females; finger length I < II < IV < III; subarticular tubercles well developed, rounded; supernumerary tubercles absent; palmar tubercle large, divided in two parts; thenar tubercle small, rounded, at the base of finger I.

Legs short, robust; knee and heel lacking tubercles; tibial and tarsal ridges absent. Foot (fig. 6 D) only slightly webbed at the base of toes in both sexes; toes lacking disks, slightly fringed; toe length I < II < V < III < IV; toes with small, lateral dermal spines in males, absent in females; subarticular tubercles well developed, rounded; supernumerary tubercles absent; a small, rounded inner, but no outer, metatarsal tubercle. Thigh length slightly less than tibia length; knee and elbow widely separated with limbs adpressed to sides of body; combined thigh and tibia length approximately 85 % of snout-vent length in males, 82 % in females, heels slightly superposed when flexed legs held at right angles to body; foot length approximately 70 % of snout-vent length in males, 64 % in females.

Skin smooth above and beneath; dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region. Anal opening not modified, lacking para-anal tubercles and glands around anus.

In preservative (70 % ETOH), color on dorsum dark brown; irregular white blotches on outer surfaces of arms and forearms; dorsal surfaces of hands and feet with small white dots

Source : MNHN, Paris

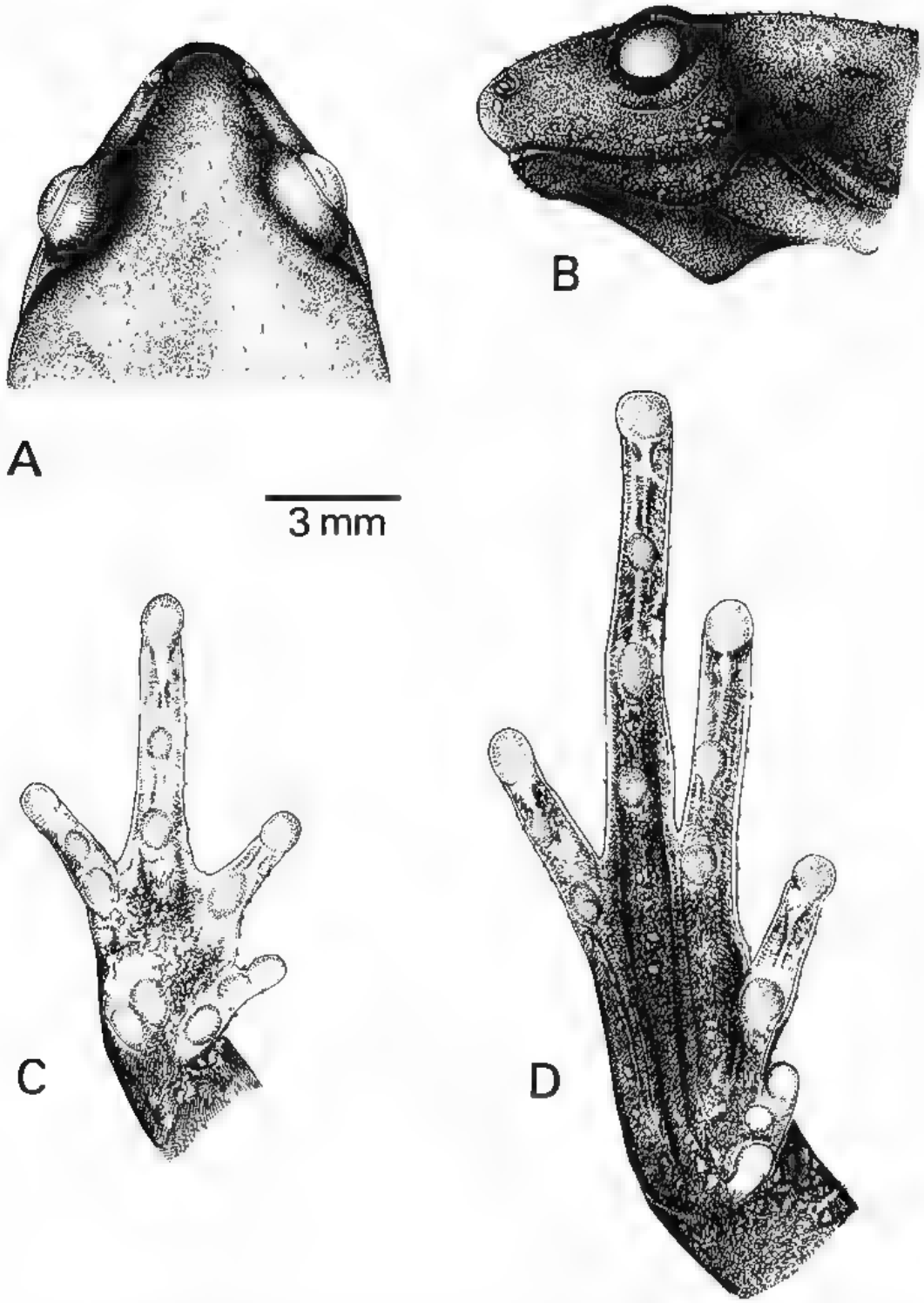

Fig. 6. – *Chiasmocleis schubarti* Bokermann, 1952 (MNRJ 17538). Dorsal (A) and ventral (B) views of head. Hand (C). Foot (D).

Source : MNHN, Paris

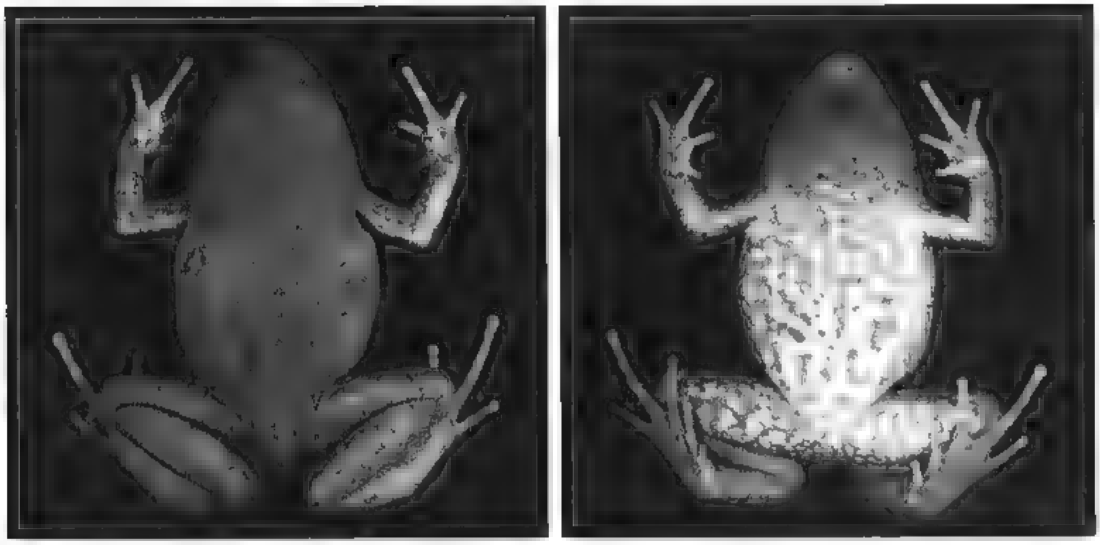

Fig. 7 – Dorsal and ventral views of *Chiasmocleis atlantica* sp. nov (MNRJ 17550, holotype).

irregularly distributed; a light longitudinal line on the posterior sides of thighs. Venter roughly marbled in dark brown and pale cream, male throat infuscated.

Variation. – Variation in measurements is presented in tab. 1. In some specimens, a light longitudinal mid-dorsal line is present.

Geographical distribution. – Known from the states of Espírito Santo and Minas Gerais in southeastern Brazil (fig. 4).

Remarks. – Scattered specimens of *C. schubarti* were collected in pitfall traps installed in low and highland forests, 40 and 800 m above sea level. Explosive reproduction, when numerous male and female specimens congregate, was observed in temporary ponds inside the forest. KASAHARA & HADDAD (1997) reported a diploid number of 24 chromosomes for this species.

Chiasmocleis atlantica sp. nov.

Holotype. - MNRJ 17550, adult male, collected at Tinguá (22°36′S, 43°26′W; ca. 40 m altitude), municipality of Nova Iguaçu, state of Rio de Janeiro, Brazil, on 27 December 1972, by C. A. G. DA CRUZ, S. D. L. RAIMUNDO, J. G. SILVA and E. IZECKSOHN.

Paratopotypes. – MNRJ 17551-17554 and EI 8940-8954, collected with the holotype; MNRJ 17549, on 30 January 1971, by J. JIM, V. C. JESUS and E. IZECKSOHN.

Diagnosis. – A medium sized species of *Chiasmocleis* diagnosed by the following combination of characters: (1) SVL 22.0-25.0 mm in males, 30.0-31.8 mm in females; (2) body trunk ovoid; (3) snout short, tip truncate in dorsal view, and slightly protruding in lateral profile; (4) hand and foot not webbed, (5) fingers and toes lacking disks, slightly fringed; (6) fingers and toes of

Source : MNHN, Paris

males with lateral dermal spines, absent in females; (7) dorsal and ventral surfaces of body and limbs with uniformly distributed dermal spines in males, absent in females except in anal region; (8) in preservative, color on dorsum uniformly brown; (9) posterior sides of thighs with a light longitudinal line; (10) venter roughly marbled in brown and pale cream.

Description. – Size medium for the genus; body trunk ovoid (fig. 7); head short, wider than long; nostrils at the tip of snout, not protuberant, and directed laterally; snout short, tip truncate in dorsal view (fig. 8 A), and slightly protruding in lateral profile (fig. 8 B); internarial distance smaller than eye to nostril distance, and larger than eye diameter; eye diameter less than eye to nostril distance; canthus rostralis slightly defined; loreal region almost vertical, slightly concave; lips not flared; eyes small, only slightly protruding; upper eyelid width one third of the interorbital space; interorbital area flat; cranial crests and occipital fold absent; postorbital fold well developed; tympanum absent; upper jaw slightly projecting beyond lower; mandible with truncate, trilobed anterior margin; tongue large, ovoid; choanae small, rounded, widely separated; a small, subgular vocal sac present.

Arms slender, lacking tubercles and crests on forearm. Hand (fig. 8 C) not webbed in both sexes; fingers lacking disks, slightly fringed; fingers with lateral dermal spines in males, absent in females; finger length I < II < IV < III; subarticular tubercles well developed, rounded; supernumerary tubercles absent; palmar tubercle large, divided in two parts; thenar tubercle small, rounded, at the base of finger I.

Legs short, robust; knee and heel lacking tubercles; tibial and tarsal ridges absent. Foot (fig. 8 D) only slightly webbed at the base of toes in both sexes; toes lacking disks, slightly fringed; toe length I < II < V < III < IV; toes with lateral dermal spines in males, absent in females; subarticular tubercles well developed, rounded; supernumerary tubercles absent; a small, oval inner, but no outer, metatarsal tubercle. Thigh length slightly less than tibia length; knee and elbow widely separated with limbs adpressed to sides of body; combined thigh and tibia length approximately 87 % of snout-vent length in males, 78 % in females; heels slightly superposed when flexed legs held at right angles to body; foot length approximately 70 % of snout-vent length in males, 67 % in females.

Skin poorly rugose above and smooth beneath; dorsal and ventral surfaces of body and limbs with uniformly distributed dermal spines in males, absent in females except in anal region. Anal opening not modified, lacking para-anal tubercles and glands around anus.

In preservative (70 % ETOH), color on dorsum uniformly brown; dorsal surfaces of hands and feet with small white dots irregularly distributed; a light longitudinal line on the posterior sides of thighs. Venter roughly marbled in brown and pale cream; male throat infuscated.

Measurements of holotype. – SVL 22.8; HL 6.9; HW 8.0; IND 2.0; END 2.0; ED 2 0; UEW 1.0; IOD 2.7; THL 10.4; TL 10.7; FL 16.7.

Variation. – Variation in measurements is presented in tab. 1. In some specimens, a light longitudinal mid-dorsal line is present.

Etymology. – The specific name, a Latin adjective, refers to the Atlantic Rain Forest of eastern Brazil

Source : MNHN, Paris

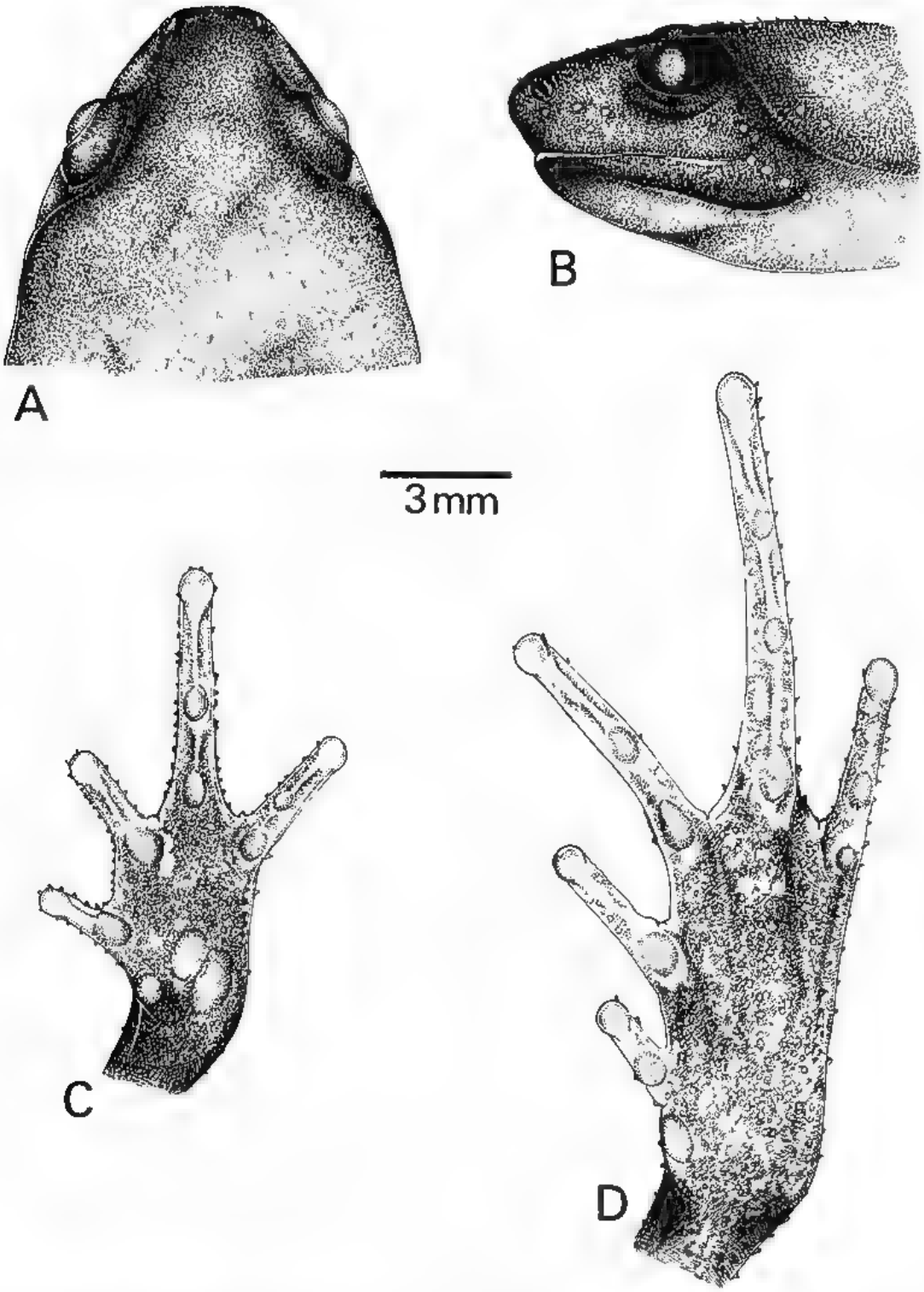

Fig. 8. *Chiasmocleis atlantica* sp. nov. (MNRJ 17550, holotype) Dorsal (A) and ventral (B) views of head. Hand (C). Foot (D).

Source : MNHN, Paris

Geographical distribution. – Known from the states of Rio de Janeiro and São Paulo in southeastern Brazil (fig. 4).

Remarks Specimens of *C. atlantica* were collected in lowland forests, at 40 m above sea level. Explosive reproduction, when numerous male and female specimens congregate, was observed in temporary ponds inside the forest.

Chiasmocleis capixaba sp. nov.

Holotype. – MNRJ 17514, adult male, collected at the municipality of Aracruz (19°59'S, 40°12'W; ca. 60 m altitude), state of Espírito Santo, Brazil, on 29 November 1995, by J. L. Gasparini.

Paratopotypes. – MNRJ 17515-17529, collected with the holotype; MNRJ 17532-17534, on 2-5 November 1994, by R. P. Bastos and J. L. Gasparini; MNRJ 17891-17895, on 29 November - 1 December 1995, by J. L. Gasparini; MNRJ 17535-17537, on 15-17 January 1996, by J. P. Pombal Jr., C. F. B. Haddad and J. L. Gasparini; EI 8955-8956, on 20-25 November 1994, by R. P. Bastos and J. L. Gasparini, CFBH 2668-2669, on 20-25 November 1994, by R. P. Bastos and J. L. Gasparini; CFBH 2685, on 23-27 January 1995, by J. P. Pombal Jr., J. L. Gasparini and C. F. B. Haddad, CFBH 2693-2695, on 27 November 1995, by J. L. Gasparini; CFBH 2701-2702, on 30 November 1995, by J. L. Gasparini; CFBH 2714, on 20 December 1995, by J. L. Gasparini.

Diagnosis. A small sized species of *Chiasmocleis* diagnosed by the following combination of characters: (1) SVL 14.7-16.5 mm in males, 17.9-21.7 mm in females; (2) body trunk elongate ovoid; (3) snout short, tip rounded in dorsal and lateral views, (4) hand not webbed, and foot webbed in both sexes; (5) fingers and toes lacking disks, extensively fringed on free parts; (6) fingers and toes of males with lateral dermal spines, absent in females; (7) dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region; (8) in preservative, color on dorsum grayish brown with gray blotches irregularly distributed; (9) posterior sides of legs with or without a light longitudinal line; (10) venter finely marbled in brown and pale cream.

Description. Size small for the genus; body trunk elongate ovoid (fig. 9); head short, approximately as broad as long; nostrils at the tip of snout, not protuberant, and directed anterolaterally; snout short, tip rounded in dorsal and lateral views (fig. 10 A-B); internarial distance equal eye diameter, smaller than eye to nostril distance; canthus rostralis rounded; loreal region oblique, slightly concave; lips not flared; eyes small, only slightly protruding; upper eyelid width smaller than half of the interorbital space; interorbital area flat; cranial crests and occipital fold absent; postorbital fold present, tympanum absent; upper jaw slightly projecting beyond lower; mandible with truncate, trilobed anterior margin; tongue large, ovoid; choanae small, rounded, widely separated; a small, subgular vocal sac present.

Arms slender, lacking tubercles and crests on forearm. Hand (fig. 10 C) not webbed in both sexes, fingers lacking disks, extensively fringed; fingers of males with lateral dermal spines, absent in females; finger length I < II < IV < III; subarticular tubercles well developed, rounded; supernumerary tubercles absent; palmar tubercle large, divided in two parts; thenar tubercle large, rounded, at the base of finger I.

Source : MNHN, Paris

Fig. 9 Dorsal and ventral views of *Chiasmocleis capixaba* sp. nov. (MNRJ 17514, holotype).

Legs short, robust; knee and heel lacking tubercles; tibial and tarsal ridges absent. Foot of male (fig. 10 D) extensively webbed, of female webbed only at the base of toes; toes lacking disks and fringed on free parts; toe length I < II < V < III < IV; toes with lateral dermal spines in males, absent in females; subarticular tubercles well developed, rounded; supernumerary tubercles absent; an oval inner, but no outer, metatarsal tubercle. Thigh length slightly longer than tibia length in males, and slightly less in females; knee and elbow widely separated with limbs adpressed to sides of body; combined thigh and tibia length approximately 79 % of snout-vent length in males, 74 % in females; heels slightly superposed when flexed legs held at right angles to body; foot length approximately 66 % of snout-vent length in males, 59 % in females.

Skin smooth above and beneath; dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region. Anal opening not modified, lacking para-anal tubercles and glands around anus.

In preservative (70 % ETOH), color on dorsum grayish brown with gray blotches irregularly distributed; loreal region dark gray; a light longitudinal mid-dorsal line on body and a similar line on the posterior sides of thighs present or absent. Ventral surfaces of body and limbs finely marbled in brown and pale cream; male throat infuscated.

Measurements of holotype. – SVL 15.5; HL 5.0, HW 5 2; IND 1.0; END 1.3; ED 1.4; UEW 1.0; IOD 1.8; THL 6.4; TL 6.6; FL 10.3.

Variation. – Variation in measurements is presented in tab. 1 Females are larger than males, with interdigital webbing less developed. The light mid-dorsal longitudinal line on body and the similar line on posterior surfaces of thighs are observed in approximately half of the examined specimens. These lines appear associated with a light line on ventral surfaces of arms and pectoral region, where it assumes a V-shape.

Source : MNHN, Paris

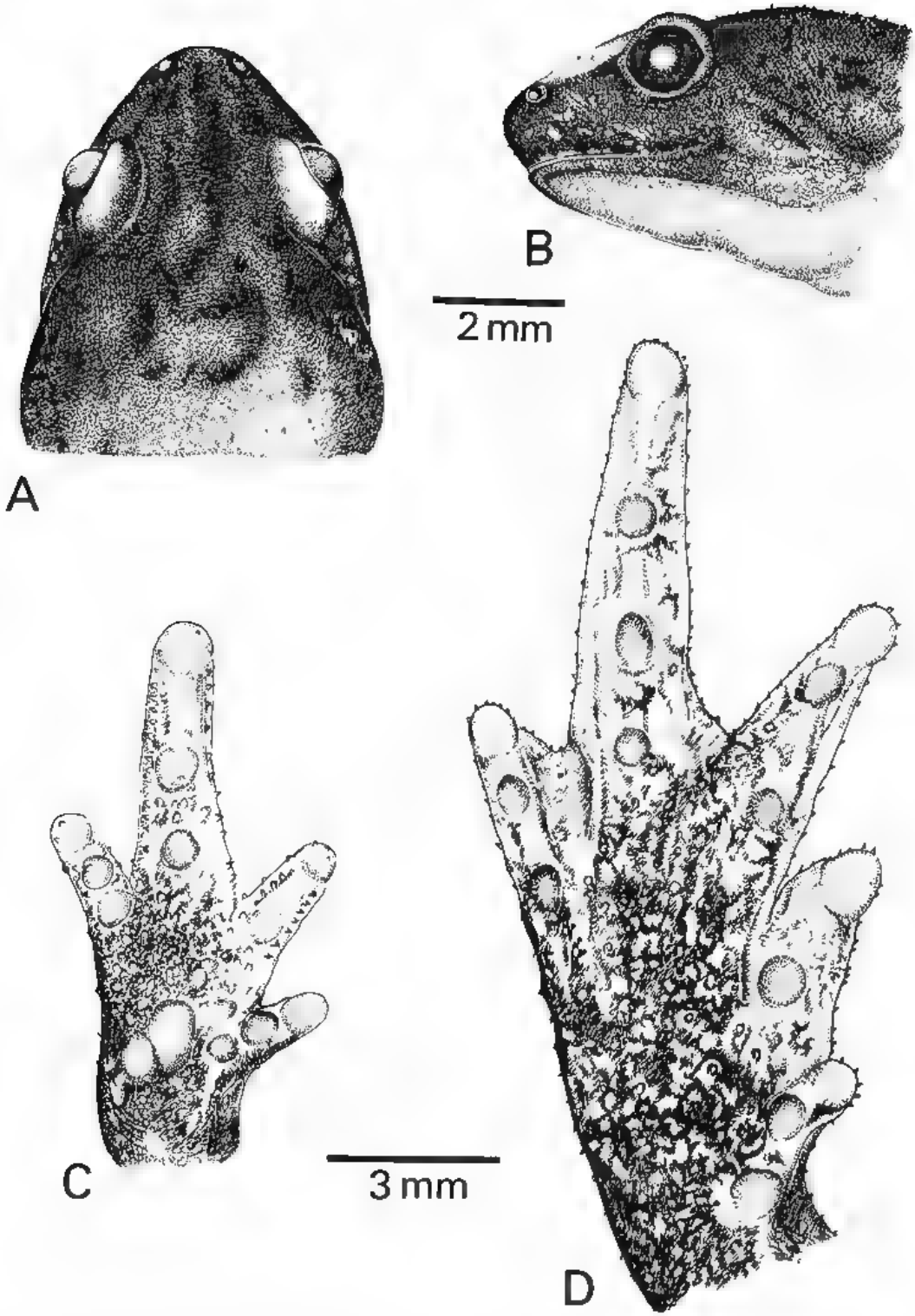

Fig 10. - *Chiasmocleis capixaba* sp. nov (MNRJ 17514, holotype) Dorsal (A) and ventral (B) views of head. Hand (C). Foot (D).

Source : MNHN, Paris

Etymology. – The name of the species, a native Brazilian word here utilized as a noun in apposition, refers to the occurrence of the species as an inhabitant of the state of Espírito Santo.

Geographical distribution. – Known only from the state of Espírito Santo in southeastern Brazil (fig. 4).

Remarks. – Scattered specimens of *C. capixaba* were collected in pitfall traps installed in lowland forests, at 60 m above sea level. Explosive reproduction, when numerous male and female specimens congregate, was observed in temporary ponds inside the forest.

Chiasmocleis carvalhoi sp. nov.

Holotype. – MNRJ 17505, adult male, collected at the Horto Florestal de Santa Cruz, municipality of Seropédica (22°44′S, 43°43′W, ca. 40 m altitude), state of Rio de Janeiro, Brazil, on 31 January 1995, by C. A. G. DA CRUZ, L. KRAUSE and G. VINCIPROVA.

Paratopotypes. – MNRJ 17506-17513 and 17565, collected with the holotype; MNRJ 17480-17504, on 2 December 1968, by E. IZECKSOHN, S. T. ALBUQUERQUE and J. SAMARÃO; EI 2096-2235, on 21 February 1964, by J. JIM and E. IZECKSOHN; EI 2236-2307, on November 1964, by J. JIM, S. T. ALBUQUERQUE, W. F. MENDONÇA and E. IZECKSOHN; EI 2308-2399, on December 1964, by J. JIM, S. T. ALBUQUERQUE, W. F. MENDONÇA and E. IZECKSOHN; EI 4381-4389, on December 1965, by S. T. ALBUQUERQUE, W. F. MENDONÇA, A. LEBEDENCO, J. JIM and E. IZECKSOHN; EI 4391-4393, on 19 October 1966, by S. T. ALBUQUERQUE, W. F. MENDONÇA and E. IZECKSOHN; EI 5332, on 17 September 1975, by E. IZECKSOHN and J. G. SILVA; EI 8937-8938, on 30 November 1979, by I. FERREIRA.

Diagnosis. – A small sized species of *Chiasmocleis* diagnosed by the following combination of characters: (1) SVL 15.5-18.3 mm in males, 16.0-22.5 mm in females; (2) body trunk ovoid; (3) snout short, tip rounded in dorsal and lateral views; (4) hand and foot not webbed in both sexes; (5) fingers and toes lacking disks, fringed, (6) fingers and toes with lateral dermal spines in males, absent in females; (7) dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region; (8) in preservative, color on dorsum brown with small white dots irregularly distributed; (9) posterior sides of legs with or without a light longitudinal line; (10) venter finely marbled in brown and pale cream.

Description. – Size small for the genus; body trunk ovoid (fig. 11); head short, slightly broader than long; nostrils at the tip of snout, not protuberant, and directed anterolaterally; snout short, tip rounded in dorsal and lateral views (fig. 12 A-B); internarial distance equal eye diameter, smaller than eye to nostril distance; canthus rostralis rounded; loreal region oblique, slightly concave; lips not flared; eyes small, slightly protruding; upper eyelid width smaller than half of the interorbital space; interorbital area flat; cranial crests and occipital fold absent; postorbital fold present; tympanum absent; upper jaw slightly projecting beyond lower; mandible with truncate, trilobed anterior margin, tongue large, ovoid; choanae small, rounded, widely separated; a small, subgular vocal sac present.

Source : MNHN, Paris

Fig. 11. – Dorsal and ventral views of *Chiasmocleis carvalhoi* sp. nov. (MNRJ 17505, holotype).

Arms slender, lacking tubercles and crests on forearm. Hand (fig. 12 C) not webbed in both sexes; fingers lacking disks, fringed; fingers of males with lateral dermal spines, absent in females; finger length I < II < IV < III; subarticular tubercles well developed, rounded; supernumerary tubercles absent; palmar tubercle large, divided in two parts; thenar tubercle small, rounded, at the base of finger I.

Legs short, robust; knee and heel lacking tubercles, tibial and tarsal ridges absent. Foot (fig. 12 D) not webbed in both sexes; toes lacking disks, fringed; toe length I < II < V < III < IV; toes with lateral dermal spines in males, absent in females; subarticular tubercles well developed, rounded; supernumerary tubercles absent; an oval inner, but no outer, metatarsal tubercle. Thigh length less than tibia length; knee and elbow widely separated with limbs adpressed to sides of body; combined thigh and tibia length approximately 82 % of snout-vent length in males, 79 % in females; heels slightly superposed when flexed legs held at right angles to body; foot length approximately 66 % of snout-vent length in males, 62 % in females.

Skin smooth above and beneath; dorsal and ventral surfaces of body and limbs with small, uniformly distributed dermal spines in males, absent in females except in anal region. Anal opening not modified, lacking para-anal tubercles and glands around anus.

In preservative (70 % ETOH), color on dorsum brown with small white dots irregularly distributed; a light longitudinal mid-dorsal line on body, and a similar line on the posterior sides of thighs, present or absent. Ventral surfaces of body and limbs finely marbled in brown and pale cream; male throat infuscated.

Measurements of holotype. – SVL 17.3; HL 5.6; HW 5.3; IND 1.3; END 1.6; ED 1.5; UEW 1.0; IOD 2.2; THL 7.2; TL 7.4; FL 11.6.

Source : MNHN, Paris

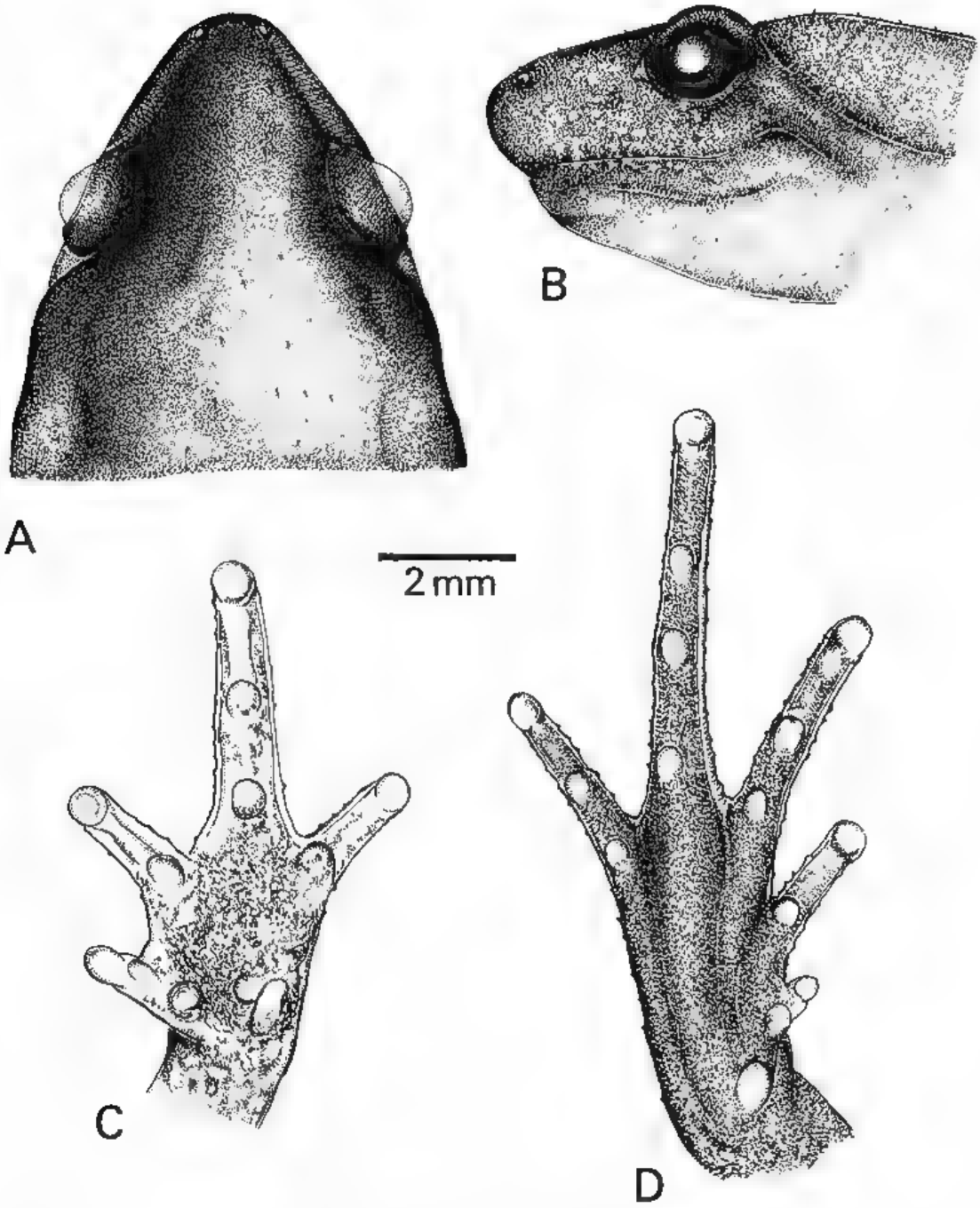

Fig. 12. – *Chiasmocleis carvalhoi* sp. nov. (MNRJ 17505, holotype). Dorsal (A) and ventral (B) views of head. Hand (C). Foot (D).

Source : MNHN, Paris

Variation. – Variation in measurements is presented in tab. 1. A light mid-dorsal longitudinal line on body and a similar line on posterior surfaces of thighs are observed in some specimens. These lines appear associated with a light line on ventral surfaces of arms and pectoral region, where it assumes a V-shape.

Etymology. – The name of the species honors the late Prof. Antenor Leitão DE CARVALHO (1910-1985), an outstanding teacher, naturalist, and herpetologist.

Geographical distribution. – Known from the states of Rio de Janeiro and São Paulo in southeastern Brazil (fig. 4)

Remarks. – Specimens of *C. carvalhoi* were collected in lowland forests, at 40 m above sea level. Explosive reproduction, when numerous male and female specimens congregate, was observed in temporary ponds inside the forest. BOGART & NELSON (1976) referred to a diploid number of 24 chromosomes for specimens identified as *C. bicegoi*, collected at Itaguaí, state of Rio de Janeiro. This reference applies to *C. carvalhoi*.

DISCUSSION

Species of *Chiasmocleis* occurring in the Atlantic Rain Forest are readily separated in two morphologically distinct groups. The first grouping involves *C. leucosticta* and *C. capixaba*, characterized by the presence of well developed webbing on the feet. The second grouping includes *C. schubarti*, *C. atlantica*, and *C. carvalhoi*, with no or only vestigial web on the feet.

Chiasmocleis leucosticta is larger than *C. capixaba* (see tab. 1), and males of the former have a fully webbed hand instead of the absence of webbing observed in *C. capixaba*. The latter species has a finely marbled venter with males presenting numerous, conspicuous lateral dermal spines on digits, whereas *C. leucosticta* presents a roughly marbled venter and small dermal spines on digits. The geographical distribution of these two species is largely disjunct (see fig. 4).

The striking webbing observed in *C. leucosticta* and *C. capixaba* is found in only one other species of the genus, *C. anatipes*, described by WALKER & DUELLMAN (1974) from Santa Cecilia, Ecuador. As noted by these authors, in the event that generic distinction should be desirable for the fully foot-webbed species, the name *Nectodactylus* Miranda-Ribeiro, 1924 (type-species *N. spinulosus*, a junior synonym of *C. leucosticta*) is available.

In the second grouping, *C. carvalhoi* is smaller than *C. schubarti* and *C. atlantica* (see tab. 1), and presents a finely marbled venter instead of the roughly marbled venter found in the other two species. *Chiasmocleis schubarti* presents the snout rounded in dorsal and lateral views, and *C. atlantica* has the snout truncate in dorsal and slightly protruding in lateral views. Moreover, males of *C. atlantica* have numerous, conspicuous lateral dermal spines on digits, whereas *C. schubarti* presents few, small lateral spines on those digits. *Chiasmocleis atlantica* and *C. carvalhoi* are sympatric, and both are disjunctly distributed in relation to *C. schubarti* and *C. leucosticta*. On the other hand, *C. schubarti* is sympatric with *C. capixaba*, and largely disjunct from *C. leucosticta* (see fig. 4).

Source : MNHN, Paris

Tab. 1 - Measurements of five species of the genus *Chiasmocleis* Méhely, 1904 from the Atlantic Rain Forest of Brazil: *Chiasmocleis leucosticta* (Boulenger, 1888), *Chiasmocleis schubarti* Bokermann, 1952; *Chiasmocleis atlantica* sp nov ; *Chiasmocleis capixaba* sp. nov.; *Chiasmocleis carvalhoi* sp. nov. *n*, number of specimens; *x*, mean; *s*, standard deviation.

	Chiasmocleis leucosticta (Boulenger, 1888)			*Chiasmocleis schubarti* Bokermann, 1952			*Chiasmocleis atlantica* sp. nov.			*Chiasmocleis capixaba* sp. nov.			*Chiasmocleis carvalhoi* sp. nov.		
Males	*n* = 12			*n* = 15			*n* = 20			*n* = 12			*n* = 15		
Characters	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*
SVL	19.0-22.8	20.0	1.26	19.2-26.3	23.5	1.87	22.0-25.0	23 1	0.80	14.7 16.5	15.6	0 58	15.5-18.3	17 1	0.76
HL	5.4-7.0	6.0	0.47	6.1-7.7	6.7	0.56	5.4-8.1	6.8	0.65	4.2-5.4	4.8	0.42	4.3-6.0	5.2	0.46
HW	5.3-6.5	5.8	0.36	5.8-8.1	7.2	0.61	6.8-8.4	7.4	0.41	4.6-5 3	4.9	0.21	4.4-6.1	5.3	0.40
IND	1.2-1.6	1.5	0.11	1.4-2.1	1.9	0.20	1.2-2.0	1.6	0.21	1.0-1.4	1.2	0.14	1.0-1.4	1.3	0.14
END	1.6-2.0	1.7	0.12	1.7-2.3	2.1	0.14	1.4-2.3	1.9	0.23	1.2-2 0	1.5	0.22	1.4-2.3	1.7	0.23
ED	1.3-1.6	1.5	0.11	1.3-2.0	1.6	0.24	1.0-1.5	1.3	0.22	1.0-1.4	1.2	0.16	1.0-1.5	1.3	0.16
UEW	0.6-1.0	0.9	0.12	0.9-1.2	1.0	0.07	1.0-1.2	1.0	0.06	0.6-1 0	0.9	0.18	0.8-1.0	0.9	0.10
IOD	2.0-2.8	2 4	0.22	2 3-3.3	2.9	0.27	2 6-3.3	2.9	0.18	1.8-2.3	2.0	0 19	2 0-2.3	2.1	0.11
THL	7.8-9.1	8.3	0.33	8.1-11.1	9.4	0.78	9.0-10.7	9.9	0.46	5.4-7.0	6.3	0.48	6.0-7.3	6.8	0.41
TL	7 8-9.6	8 8	0.47	8.7-11.2	10.1	0.74	9.8-11.4	10 6	0.42	5.1-7 0	6.2	0.58	6 6-7.8	7.3	0.32
FL	11.9-15.6	13.4	0.88	14.5-17.5	15.9	1.04	11.7-17.8	16.5	1.31	9.5-11.2	10.3	0.45	10.0-12.6	11.3	0 78
Females	*n* = 15			*n* = 12			*n* = 3			*n* = 16			*n* = 15		
Characters	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*	Range	*x*	*s*
SVL	21.8-25.5	23.6	1.15	20 4-34.5	28.7	4.60	30.0-31.8	30.6	1.04	17.9-21.7	20.2	1.01	16.0-22.5	20.2	1.93
HL	5 7 7 5	6 8	0.52	5 6-8 2	7 4	0 87	6 6-7.8	7.3	0.61	4.4-6 7	5.7	0.64	4.2-6.6	5.5	0.66
HW	5.7-7.4	6.4	0.45	6.2-10.2	8.0	1.15	8.1-8.2	8.1	0.06	5.4-6.2	5.7	0.22	5.0-6.4	5.6	0.40
IND	1.3-2 0	1 6	0.15	1 6-2 2	1 9	0.23	1 8-2.4	2.1	0.31	1.0-1.4	1.2	0.15	1.2-1 6	1 4	0.15
END	1.7-2 2	1.9	0.14	2.0-2.6	2 2	0.19	1.9-2.4	2.1	0.25	1.4-1 8	1.6	0.10	1.4-2.0	1.8	0.20
ED	1.4-2.0	1.6	0.18	1.4-2.0	1.8	0.24	1.6-2.0	1.8	0.21	1.0-1.9	1.3	0.21	1.0-2.0	1 4	0.30
UEW	0.7-1 3	1.0	0 12	0.9-1.4	1.1	0.16	1 1-1 4	1.3	0.15	0.7-1 0	0.9	0.09	0.8-1 0	1 0	0.07
IOD	2.5-3.0	2.7	0.18	2.7-3.7	2.2	0.29	3.6-3.7	3.7	0.06	2.0-2.9	2.4	0.20	2.1-3.0	2.4	0.28
THL	9 0 10 5	10.0	0 42	8 4-13 5	11.1	1 51	11 5-12 3	11 8	0.44	6 7-8.0	7.3	0.43	6.2-9.1	7.6	0.73
TL	9 3-11 0	10.3	0 46	9 4-13 8	11.9	1.39	11.8-12.8	12 3	0.50	6.4-8.3	7.5	0.61	6.5-9 1	7.9	0.70
FL	15.1-17.4	16.3	0.77	14.1-20.7	18.1	2 19	20.0-21.2	20.5	0.61	10.6-12.8	12.0	0.77	9.9-14.2	12.3	1.21

Source : MNHN, Paris

Résumé

L'étude taxinomique des espèces du genre *Chiasmocleis* de la Forêt Atlantique brésilienne est presentée. *Chiasmocleis bicegoi* Miranda-Ribeiro, 1920 est mise en synonymie de *C. albopunctata* (Boettger, 1885), une espèce distribuée hors des limites géographiques considérées dans le présent travail. La synonymisation de *C. urbanae* Bokermann, 1952 avec *C. leucosticta* (Boulenger, 1888) est confirmée. Une description détaillée de *C. leucosticta* et de *C. schubarti* Bokermann, 1952 est fournie. Trois nouvelles espèces sont décrites.

Resumo

O estudo taxonômico das espécies do gênero *Chiasmocleis* que ocorrem na Floresta Atlântica do leste do Brasil é apresentado. *Chiasmocleis bicegoi* Miranda-Ribeiro, 1920 é sinonimizada com *C. albopunctata* (Boettger, 1885) e considerada fora da delimitação geográfica abrangida pelo presente trabalho. A sinonimização de *C. urbanae* Bokermann, 1952 com *C. leucosticta* (Boulenger, 1888) é reafirmada. *Chiasmocleis leucosticta* e *C. schubarti* Bokermann, 1952 são redescritas e três espécies novas são descritas.

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APPENDIX 1

LIST OF SPECIMENS EXAMINED

Chiasmocleis leucosticta. – BRAZIL: *São Paulo*: Casa Grande (MNRJ 17564), Ribeirão Branco (MNRJ 17900-17904, CFBH 268, 2136, 2229-2232, 2234, 2236-2237, 2239-2243, 2245-2247, 2251-2652; EI 8957-8958); Ilha de São Sebastião (MZUSP 9033, holotype of *Chiasmocleis urbanae*); *Santa Catarina* Corupá (MNRJ 525, 5582, syntypes of *Nectodactylus spinulosus*); Santa Luzia e Araujos (MNRJ 17563).

Chiasmocleis schubarti. – BRAZIL: *Espirito Santo*: Linhares, Córrego Juncado (MZUSP 2309, holotype); Linhares, Sooretama (EI 2095; MNRJ 17548); Santa Teresa (EI 8939); Aracruz (MNRJ 17538, 17539, 17542-17545, 17546, 17547, 17896-17899; CFBH 2667, 2703-2704, 2710-2713; EI 8959-8960); *Minas Gerais*: Marliéria, Parque Estadual do Rio Doce (MNRJ 17883-17890; UFV 2600-2601, 2603-2608, 2610, 2612-2615, 2617-2618).

Chiasmocleis atlantica. – BRAZIL: *Rio de Janeiro*. Nova Iguaçu, Tinguá (MNRJ 17550, holotype; MNRJ 17549, 17551-17554, paratypes; EI 8940-8954, paratypes); *São Paulo*: Ubatuba, Picinguaba (SPCS 5605-5607).

Chiasmocleis capixaba. – BRAZIL: *Espirito Santo*. Aracruz (MNRJ 17514, holotype, EI 8955-8956, paratypes; MNRJ 17515-17529, 17532-17534, 17535-17537, 17891-17895, paratypes; CFBH 2668-2669, 2685, 2693-2695, 2701-2702, 2714, paratypes).

Chiasmocleis carvalhoi. – BRAZIL *Rio de Janeiro*: Seropédica, Horto Florestal de Santa Cruz (MNRJ 17505, holotype; MNRJ 17480-17504, 17506-17513, 17565, paratypes, EI 2096-2235, 2236-2307, 2308-2399, 4381-4389, 4391-4393, 5332, 8937-8938, paratypes); Arraial do Cabo, Ilha de Cabo Frio (MNRJ 17555-17562); Duque de Caxias (MNRJ 17566-17567, 17568-17571, 17572, 17573-17575, 17576); Niterói (MNRJ 17577, 17578), Nova Iguaçu, Tinguá (EI 4378, 4379, 4380, 4390, 4394); *São Paulo*: Ubatuba (CFBH 1322, 1571); Ubatuba, Picinguaba (SPCS 5608-5610, 5612-5613, 5617, 5620-5621).

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Erythrocyte size and ploidy determination in green toads (*Bufo viridis* complex) from Middle Asia

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The sizes of erythrocytes in blood smears of green toads (*Bufo viridis* complex) from diploid and tetraploid Middle Asian populations (Iran, Turkmenistan, Uzbekistan and Kyrgyzstan) were compared by measuring their projection areas. Significant differences in the average values of erythrocyte areas between diploid and tetraploid toads were found. In some populations the values are in the overlapping range between both levels of ploidy. Erythrocyte areas variability, in relation to age, body size and altitude of the habitat, are discussed in the light of literature data on green toads and other polyploid anurans. The staining of erythrocytes with the Feulgen reaction and the microdensitometrical determination of the DNA content are described and considered to be an unequivocal method which does not require to kill the animals.

INTRODUCTION

Middle Asia (for ecogeographical definition, see WALTER & BRECKLE, 1986: 233, 275) is inhabited by diploid and tetraploid green toads (*Bufo viridis* complex; for taxonomic survey, see: ROTH, 1986, KUZMIN et al., 1988; STÖCK, 1995, 1997). In order to name diploid and polyploid forms from Middle and Central Asia which are all quite closely related to *Bufo viridis*, we use the term "*Bufo viridis* complex". This excludes some species (e.g., *Bufo calamita* or *Bufo surdus*) belonging to INGER's (1972) "*Bufo viridis* group", which was defined without information about the existence of polyploid forms. Since the discovery of tetraploid forms (MAZIK et al., 1976), the ploidy determination in these toads was based first on cytogenetic, cytophotometric and electrophoretic investigations, and concerned therefore only a quite low number of animals from each locality (BACHMANN et al., 1978; PISANETS, 1978; TOKTOSUNOV, 1984; KRJUKOV et al., 1985; BORKIN et al., 1986*a-b*, 1995; ORLOVA & UTEŠEV, 1986; ROTH & RÁB, 1986, 1987; WHU & ZHAO, 1987; KUDRJAVCEV et al., 1988; KUZMIN et al., 1988; MEŽŽERIN & PISANETS, 1990, 1995*a-b*; PISANETS, 1991, 1992*a-b*). On the other hand, in addition or exclusively, external morphological characters (which are sometimes misleading: ROTH, 1986, STÖCK, 1995, 1997) or form and size of the clutch (a questionable character too: KUZMIN, 1995: 94; STÖCK, unpublished) were used when the laborious methods mentioned above had to be limited to a few specimens or to avoid killing animals (ATAEV, 1987; PISANETS, 1987, 1992*a-b*; KUZMIN et al., 1988; TOJMASTOV, 1989, MEŽŽERIN & PISANETS, 1990; MEYER, 1991).

Source : MNHN, Paris

The aim of the present study was to develop additional methods for ploidy determination of many animals and suitable in field examinations in order to improve our knowledge about diploid and tetraploid green toads. Two methods were explored in this respect: (1) the simple microscopic measurement of projection areas of erythrocytes; (2) the microdensitometric measurement of DNA amounts.

MATERIAL AND METHODS

SPECIMENS

Samples were taken from Middle Asian animals in four diploid ($n = 36$) and six tetraploid populations ($n = 98$; see tab. 1, fig. 1) in spring 1993, 1994 and 1995 and autumn 1993 (for comparison, 13 animals from Halle/Saale, Germany, were used; permission by District Presidium Halle, 18.01.95). In these populations other investigations, such as morphological, bioacoustical and karyological analyses, were made (STÖCK, 1995, 1997, 1998). The skin of the tip of the fourth digit of each toad was scratched with a scalpel. This intervention seems to be of a little damage for an animal: in terrarium we observed the normal feeding of a toad as soon as a few seconds after manipulation and the wound was closed in one or two days. A small blood dose was used to make a blood smear on a clean microscopic slide (a single sample from each toad); in method (2) below, a "control smear" from an animal of known ploidy was made on the other end of the slide. The blood smears must not be polluted with skin mucus.

METHODS

Choice of methods

(1) In the past, the measurement of the size of cell nuclei took a leading part in the determination of polyploid individuals and species in Amphibia (reviews in: WEIß, 1954; HERTWIG et al., 1958). Moreover, relatively early, the empirically known ratio of genome size (DNA amount) to cell size was used for identification of polyploid amphibians, and erythrocytes were favoured because of their simple shape (reviews: GÜNTHER, 1977; AUSTIN & BOGART, 1982). Until today, some comparative studies (OLMO & MORESCALCHI, 1975, 1978; KURAMOTO, 1981; HORNER & MACGREGOR, 1983) verified that, both in Urodela and in Anura, there is a direct linear correlation between the DNA content and the nuclear volume, cell volume and cell surface of erythrocytes. BACHMANN et al. (1978) and KUDRJAVCEV et al. (1988) referred to greater cells of polyploid Middle Asian green toads, but with no statistical demonstration. HERRMANN (1989) suggested a micromorphological discrimination of diploid and tetraploid green toads using Raster Electron Microscope for distinguishing surface structures of the skin, but this author did not exactly determine the ploidy of his specimens.

Source : MNHN, Paris

Tab. 1. – Populations, numbers (*n*) and localities of specimens of green toads (*Bufo viridis* group) investigated.

Population Ploidy (diploid: 2 n = 22 tetraploid: 4 n = 44)	Abbreviation used in fig. 1	*n*	Localities of the populations, altitudes, dates of investigations
Bami 2 n = 22	a	23	38°37'N 56°38'E, Turkmenistan, Kysyl-Arvatskii Rayon, Kopet-Dag Mountains, valley approx. 25 km SE the station, S of the pass, alt. 750 m, March 1994
Bacharden 2 n = 22	b	3	38°14'N 57°31'E, Turkmenistan, Ashchabadskaya Oblast', approx. 10 km W Kelyata, alt. 500 m, April 1994
NE Iran 2 n = 22	c	9	37°38'N 55°29'E, frontier district near Turkmenistan, approx. 50 km NE Gonbad-e-Kāvūs, alt. 250 m, April 1994
Bishkek 2 n = 22	k	1	42°53N, 74°46'E, Kyrgyzstan, Bishkek, Botanical Garden, September 1993
Danata 4 n = 44	d	17	39°06'N 55°06'E, Turkmenistan, Ashchabadskaya Oblast', stream 2-4 km SE and warm spring approx. 4 km SE, alt. 200 m, April 1994
Bol'shoi Balkhan 4 n = 44 (and 2 n = 22?)	e	13	39°43'N 54°29'E, Turkmenistan, Nebit-Dagskii Rayon, Bol'shoi Balkhan Mountains, northern slope, approx. 15 km S Oglanly village, alt. 500 m, April 1994
Nuratau 4 n = 44	f	36	40°35'N 66°30'E, Uzbekistan, Dzhizakhskaya Oblast', Rayon Farish, Nuratau Nature Reserve, northern slope, alt 300-1600 m, May 1995
Tashkent 4 n = 44	g	6	41°16'N 69°13'E, Uzbekistan, alt. 450 m, April 1995
Chatkal 4 n = 44	h	5	41°35'N 70°07'E, Uzbekistan, 80 km E Tashkent, Chatkal Nature Reserve, 5 km E Burchmulla, alt. 900 m, April 1995
Issyk-Kul' 4 n = 44	i	21	42°29'N 76°20'E, Kyrgyzstan, northern bank near village Sary-Kamysh, alt 1670 m, April 1995

Source : MNHN, Paris

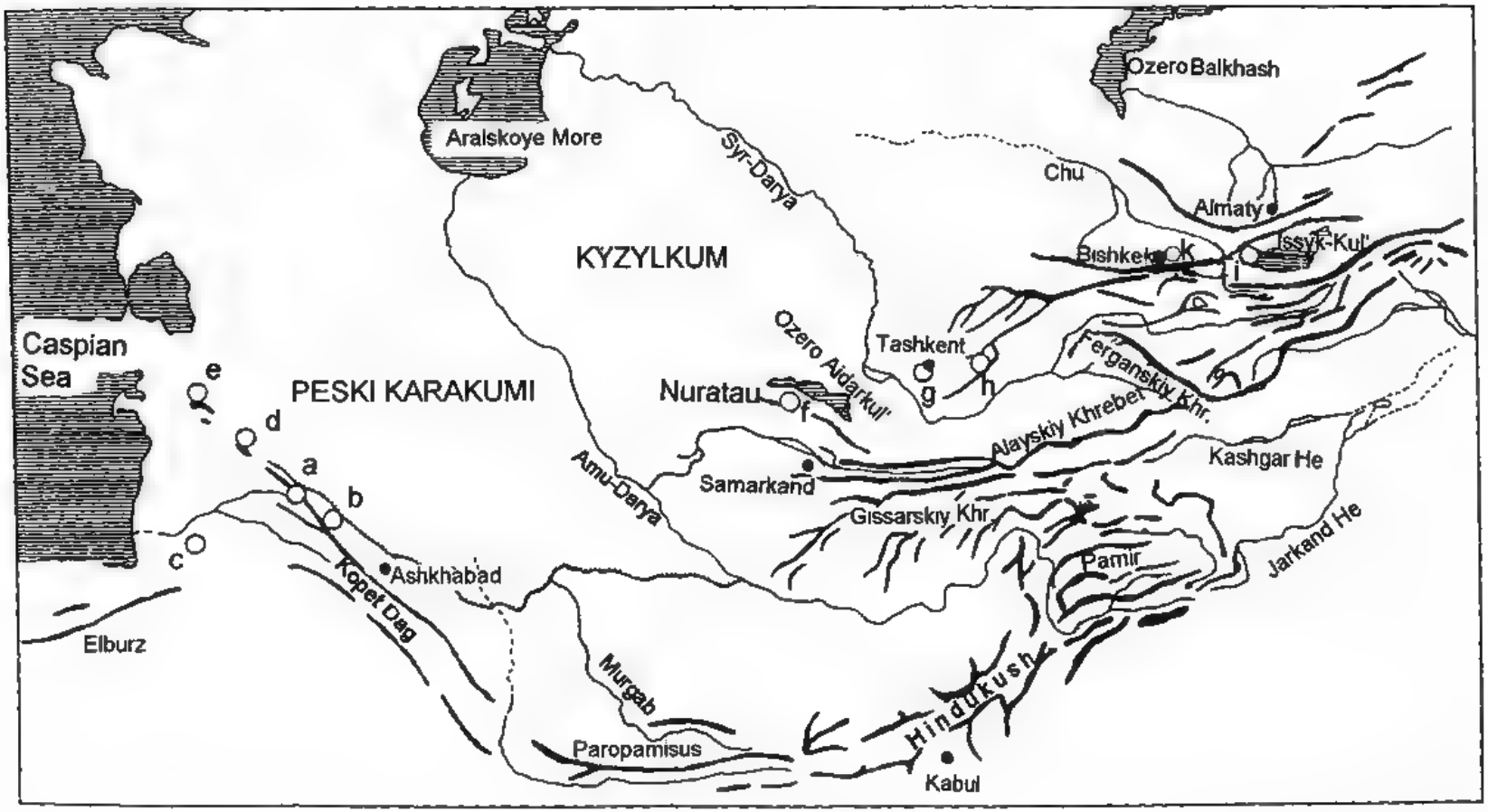

Fig. 1. Localities of the populations of green toads (*Bufo viridis* complex) investigated. Letters: see tab. 1. Map by Katrin SCHNEIDER & Matthias STÖCK, modified from WALTER & BRECKLE (1991: 275) For spelling of geographical terms, see ANONYMOUS (1993).

Source : MNHN, Paris

Tab. 2. - Average values of erythrocyte areas from populations investigated and of exemplary specimens and larvae which were karyologically investigated (counting of chromosomes). Numbers of toads involved in microdensitometry. For comparison a diploid population from central Europe (Halle/Saale) was added. *, values in the overlapping range between diploid and tetraploid populations.

Population (altitude)	Halle / Saale (110 m)	NE Iran (250 m)	Bacharden (500 m)	Bami (750 m)	Bishkek (850 m)	Bol'shoi Balkhan (500 m)	Danata (200 m)	Nuratau (300-1600 m)	Tashkent (430 m)	Chatka (1900 m)	Issyk-Kul' (1600 m)
Ploidy	2 n = 22	2 n = 22	2 n = 22	2 n = 22	2 n = 22	4 n = 44 (?)	4 n = 44	4 n = 44	4 n = 44	4 n = 44	4 n = 44
n of toads (blood smears)	13	9	3	23	1	13	17	36	6	5	21
Average erythrocyte projection areas (μm^2) - of populations	231 89	264.48	243.79	283 49	[279 78]	305.80*	340 12	334.81	323.56	333.56	374.73
- of toads karyologically investigated	-	270.49	-	283 64	279.78	292.49* 322.26	330 55 367 94	311 14* 317.28*	321 12	325.49	329.10 363 67
n of toads involved in microdensitometry	1	3	1	-	-	-	-	2	-	1	2
n of larvae karyologically investigated	2	-	2	2	-	-	2	3	2	-	-

Tab. 3. - LSD test. Significance level: $P < 0.05$. *, significant differences (shown in the lower triangle).

Population	Halle	NE Iran	Bacharden	Bishkek	Bami	B Balkhan	Tashkent	Chatkal	Nuratau	Danata	Issyk Kul'
Halle											
NE Iran	*										
Bacharden	*										
Bishkek	*										
Bami	*	*									
B. Balkhan	*	*	*		*						
Tashkent	*	*	*	*	*						
Chatkal	*	*	*	*	*	*					
Nuratau	*	*	*	*	*	*					
Danata	*	*	*	*	*	*					
Issyk-Kul'	*	*	*	*	*	*	*	*	*	*	

Source : MNHN, Paris

(2) "The use of a scanning and integrating microdensitometer that is capable of making large numbers of accurate and reproducible readings from Feulgen-stained nuclei is probably the best way of determining the DNA content of cell nuclei" (HORNER & MACGREGOR, 1983), but time and condition of the main influencing steps of the Feulgen reaction (fixation and hydrolyses) "must be determined experimentally at the start of each and every programme of microdensitometry (...) for different tissues" (MACGREGOR & VARLEY, 1983: 233).

Description of methods

(1) Following GÜNTHER (1977) and POLLS PELAZ & GRAF (1988), the blood smears were only air dried. The microscopically visible projection areas of 30 or 50 randomly chosen, normally shaped erythrocytes were measured directly. We used the image analyses system CYDOK (Fa. Hilgers, see below) in combination with a transmission light microscope (ZETOPAN, Reichert, Vienna; 25 × enlarging objective) in the Hautklinik at the Martin-Luther-University. The minimum, maximum, average value and standard deviation of the areas were calculated. Photographs were taken using Orowopan 25 (15 DIN).

(2) Some air dried blood smears from field investigations which were stored more than one year and some few fresh blood smears (diploid: $n = 5$; tetraploid $n = 5$; tab. 2) from animals living in a terrarium were fixed according to HORNER & MACGREGOR (1983) for 10 min in fresh, ice cold methanol/glacial acetic acid (3/1). Slides were then processed through the Feulgen reaction using the method of SWIFT (1955). Hydrolyses for 18 min in 5 M HCl (Merck 1.09911) at 18°C, staining in Schiff's reagent (Merck 9034) for 90 min and washing in several changes of sulphite rinse (10 ml 1 N HCl + 10 ml 5 % solution of Natriummetabisulphite + 180 ml water) followed respectively HORNER & MACGREGOR (1983) and MINZUNO & MACGREGOR (1974). The stained preparations were dehydrated carefully through 95 % and 100 % ethanol and xylene and then mounted in a synthetic mountant and covered with coverglasses. One or two hundred nuclei were measured, in comparison with the samples of known ploidy used as a standard. We used the image analyses system CYDOK (Fa. Hilgers) and a transmission light microscope with a 40 × enlarging objective at a wavelength of 539 nm. The microscopic picture is transmitted life to a screen (enlarging factor approximately 1800 ×) via video-camera (effective size of the picture 420 × 420 pixels) The Feulgen dye content (the DNA amount) of a nucleus randomly chosen by the operator (mouse click) was determined by measuring the integrated optical density in the area covered by this nucleus.

RESULTS

ERYTHROCYTE AREAS

Erythrocyte areas in toads of both levels of ploidy

Erythrocytes are easily distinguishable from other blood cells by their ellipsoid shape. KHALIL & ELFEKY (1986) provided a detailed description of blood cells morphology in *Bufo viridis* from Egypt.

Source : MNHN, Paris

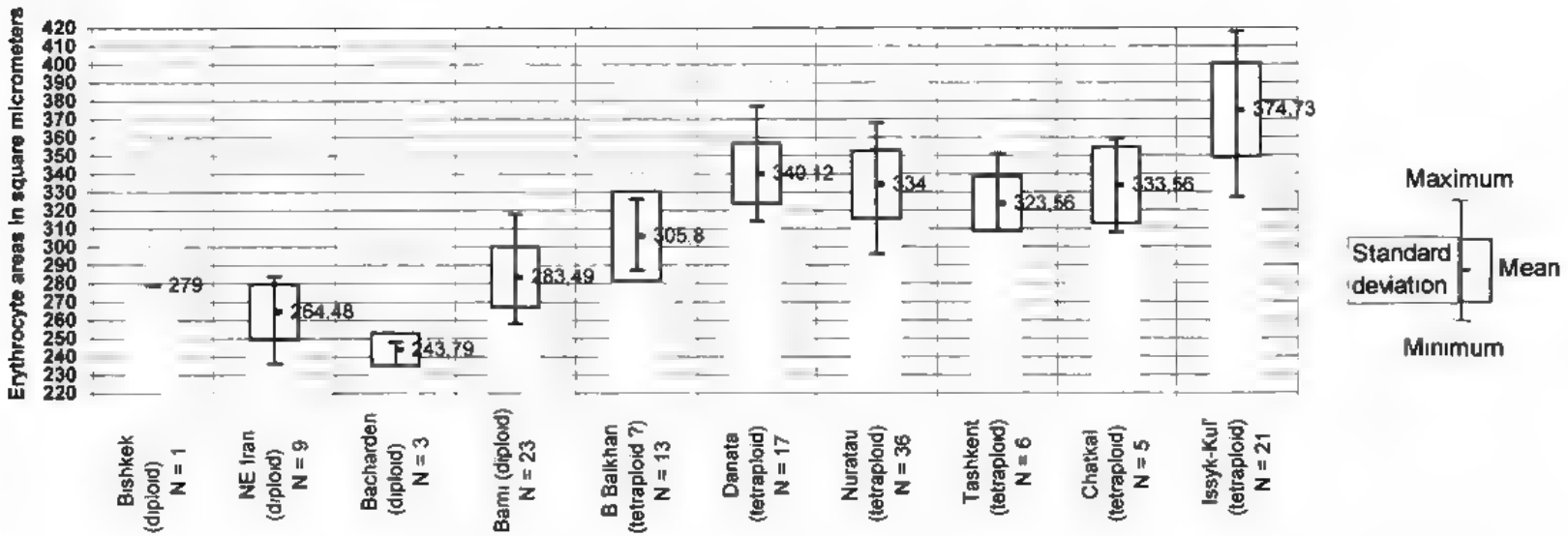

Fig. 2. – Minimum, maximum, standard deviation and average value (mean) of erythrocyte size in diploid and tetraploid green toad populations.

Source : MNHN, Paris

The average value of all diploid Middle Asian green toads investigated was 277.64 μm^2 (standard deviation, $s = 19.28$ μm^2), while in tetraploid toads it was 338.13 μm^2 ($s = 27.71$ μm^2). A Student t test for statistical comparison of the two means showed a significant difference with $P < 0.0001$. The variation range of all diploid individuals (235 μm^2 to 318 μm^2), whose number is also smaller, is distinctly smaller than that of tetraploid toads (287 μm^2 to 418 μm^2); this was especially caused by the values measured in the population Issyk-Kul' (see below).

Figure 2 displays the average value, the standard deviation and the variation range in each population. Average projection areas in the populations Danata, Nuratau, Tashkent and Issyk-Kul' in which exemplary specimens were identified as tetraploid were mostly larger than 310 μm^2. On the contrary, the diploid populations Bami, NE Iran and Bacharden, as well as the diploid specimen from Bishkek, exhibited values mostly smaller than 310 μm^2. The distinction of diploid and tetraploid green toads from these populations is possible with an error rate of only about 3.3 %: among 36 diploid toads, only 1 individual displayed a value higher then 310 μm^2; among 85 tetraploids, 3 had a value lower than 310 μm^2. The values of the average erythrocyte area in the population Bol'shoi Balkhan were situated in the overlapping range between diploid and tetraploid toads values and are therefore difficult to use for identification. In this population, the two investigated animals were identified as tetraploid (erythrocyte areas 292 μm^2 and 322 μm^2; see tab. 2), but the occurrence of diploid or triploid specimens in the sample could not be fully excluded (see *Discussion*).

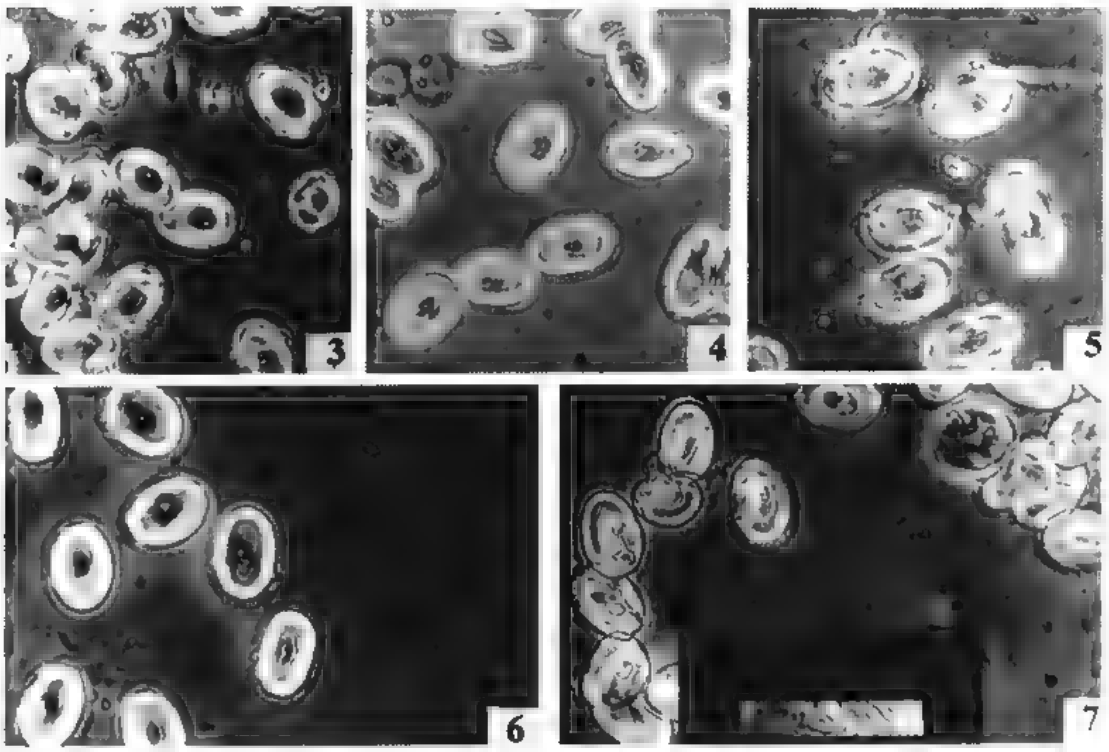

Fig. 3 7. Sections of blood smears from two diploid and tetraploid green toads. (3) Diploid individual from NE Iran; average value of erythrocyte projection area: 258.53 μm^2. (4) Diploid individual from Bishkek; 279 78 μm^2. (5) Tetraploid individual from Danata; 367 94 μm^2 (6) Tetraploid individual from Issyk-Kul'; 363.67 μm^2. (7) Tetraploid individual from Nuratau, 311.14 μm^2.

Source : MNHN, Paris

Taking the population Bol'shoi Balkhan into consideration, there is an overlapping range between 287 μm^2 (minimal average value of tetraploids; $n = 98$) and 318 μm^2 (maximal average value of diploids; $n = 36$). This range includes the values of eight diploids from the population Bami and of 21 tetraploids (10 from Bol'shoi Balkhan, $n = 13$; 6 from Nuratau, $n = 36$; 2 from Tashkent, $n = 6$; 2 from Chatkal, $n = 5$; 1 from Danata, $n = 17$). Therefore this method only allowed to classify unambiguously 77.8 % of diploid and 78.6 % of tetraploid green toads. Figures 3-4 show sections of blood smears of two diploid specimens from the populations NE Iran and Bishkek. Examples of tetraploid specimens from the populations Danata, Nuratau and Issyk-Kul' are to be seen in fig. 5-7.

Erythrocyte areas of the individuals

In all specimens investigated, a high variability of erythrocyte areas was found. Minima and maxima in a sample (one sample measured from each toad) were up to 25 % above or below the average value. However, despite the resulting fluctuation in standard deviation ($s = 25\text{-}35\ \mu m^2$), we consider that the measurement of 30 or 50 erythrocyte areas gives a good representation of the erythrocytes of an individual.

Erythrocyte areas of the populations

When enough animals could be tested, the variation range was found to vary between populations: from 39 μm^2 between minimum and maximum value in Bol'shoi Balkhan (12 % of the average value; $n = 13$), through 48 μm^2 in NE Iran (18 % of the average value; $n = 9$), 60 μm^2 in Bami (21 % of the average value; $n = 23$), 63 μm^2 in Danata (18,5 % of the average value; $n = 17$), 72 μm^2 in Nuratau (21,5 % of the average value, $n = 36$), and up to 91 μm^2 in Issyk-Kul' (24,3 % of the average value; $n = 21$; see fig. 2). Variation covers a similar range in the diploid and tetraploid populations. Although in some cases the number of specimens is too low to be statistically meaningful, distinct differences of the average values between some populations can be noticed (see tab. 2). This is clear especially in the population Issyk-Kul' whose average value of 374 μm^2 is considerably higher than in all other tetraploid populations studied. To compare the average values of all populations, an analysis of variance was performed. The average value of each diploid population was significantly different from each tetraploid population (Multiple Range test/LSD test; $P < 0.05$; see tab. 3). Furthermore it was found that the average value of erythrocyte areas in the population Issyk-Kul' is significantly different from every other diploid and tetraploid population. While the tetraploid populations Danata, Nuratau, Tashkent and Chatkal seem to be quite homogeneous (but sample sizes in Tashkent and Chatkal are small), and exhibit no significant differences between each other, the population Bol'shoi Balkhan occupies, as expected, a special position. Its average value is different from those of the populations Danata, Nuratau and Chatkal (but not from Taskent), and also from each diploid population (NE Iran, Bacharden, Bami). The average value of the population Halle from Central Europe differs from each average value of the Middle Asian populations. With 231.89 μm^2, it is smaller than the minimal values of the Middle Asian populations (NE Iran: 264 48 μm^2; Bacharden: 243.79 μm^2).

Relationship between body size, age and erythrocyte size

A ratio of erythrocyte area to snout-urostyle length within a population was considered useful for testing a dependence of the parameter on age or body size respectively. Figure 8

Source : MNHN, Paris

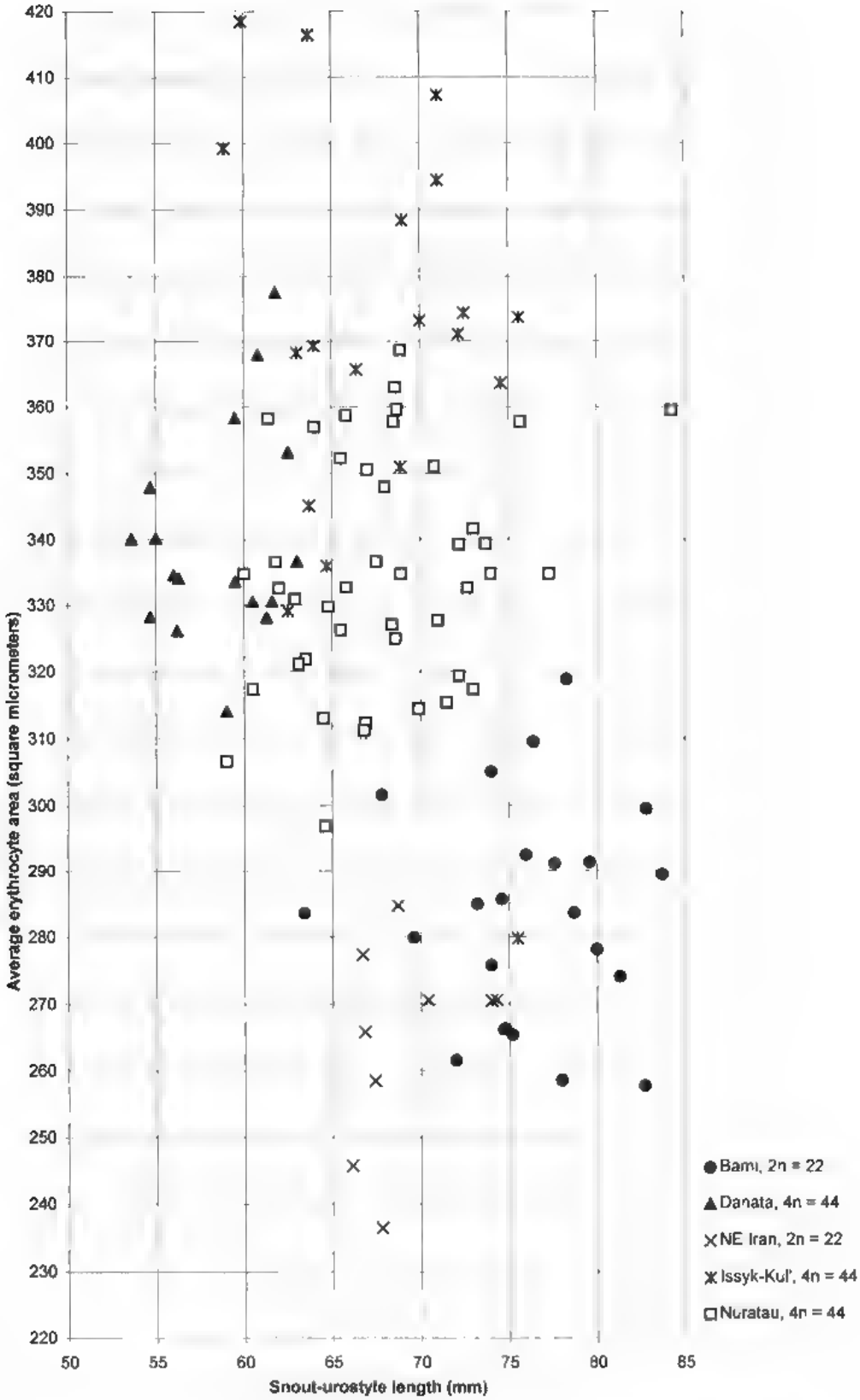

Fig. 8 Erythrocyte size and body size in two diploid and three tetraploid green toad populations.

Source : MNHN, Paris

shows this relation for two diploid and three tetraploid populations. Body size, although exhibiting high variation, showed no correlation with erythrocyte size. Therefore, body size does not affect the identification of ploidy levels of mature animals. However, the additional investigation of some tetraploid juveniles (snout-urostyle length 3 to 5 cm) from the population Issyk-Kul' showed values (327, 327 and 342 μm^2) which were at the lower border of the values within this population. Four juveniles (snout-urostyle length 4.5 to 5 cm) from the population Nuratau had similarly low values (290, 306, 316 and 317 μm^2); a juvenile from Danata (3.5 cm) had the second smallest value within this population.

Erythrocyte areas, sex and altitude of the habitat

There is no noticeable sexual dimorphism in this character: within the populations, the values of the (underrepresented) females were uniformly distributed among the values of males. No relationship was found between erythrocyte area and altitude (see tab. 2) of toad habitats.

Microdensitometrical measurements

Stored, air dried blood smears

The nuclei in air dried blood smears which were stored without freezing some weeks or months were not suitable for quantitative staining with the Feulgen reaction. The older the sample was, the less it could be stained.

Fresh fixed blood smears

On the contrary, fresh fixed blood smears exhibited very exact results. Figure 9a shows as an example the frequency distribution of the DNA amounts measured in 100 erythrocyte nuclei of a diploid (DNA index I) specimen from the population NE Iran. Figure 9b demonstrates the DNA amounts of 200 nuclei of a tetraploid (DNA index II) from the population Issyk-Kul'. Since the total DNA amount of a nucleus is measured, what could in principle result also from an aneuploid chromosome number, in such measurements the DNA amount of a diploid nucleus is named as DNA index 1 and relative values were analysed.

Discussion

Erythrocyte areas

Method

The measurement of erythrocyte areas yields useful results for distinguishing between diploid and tetraploid green toads. Combined with the hitherto presented morphological characters (Pisanets, 1978; Pisanets & Ščerbak, 1979; Kuzmin, 1995) or with multivariate analyses of morphometrical data (Stöck, 1997), the method offers new opportunities for the determination of Middle Asian green toads. In spite of this study including only one diploid

Source : MNHN, Paris

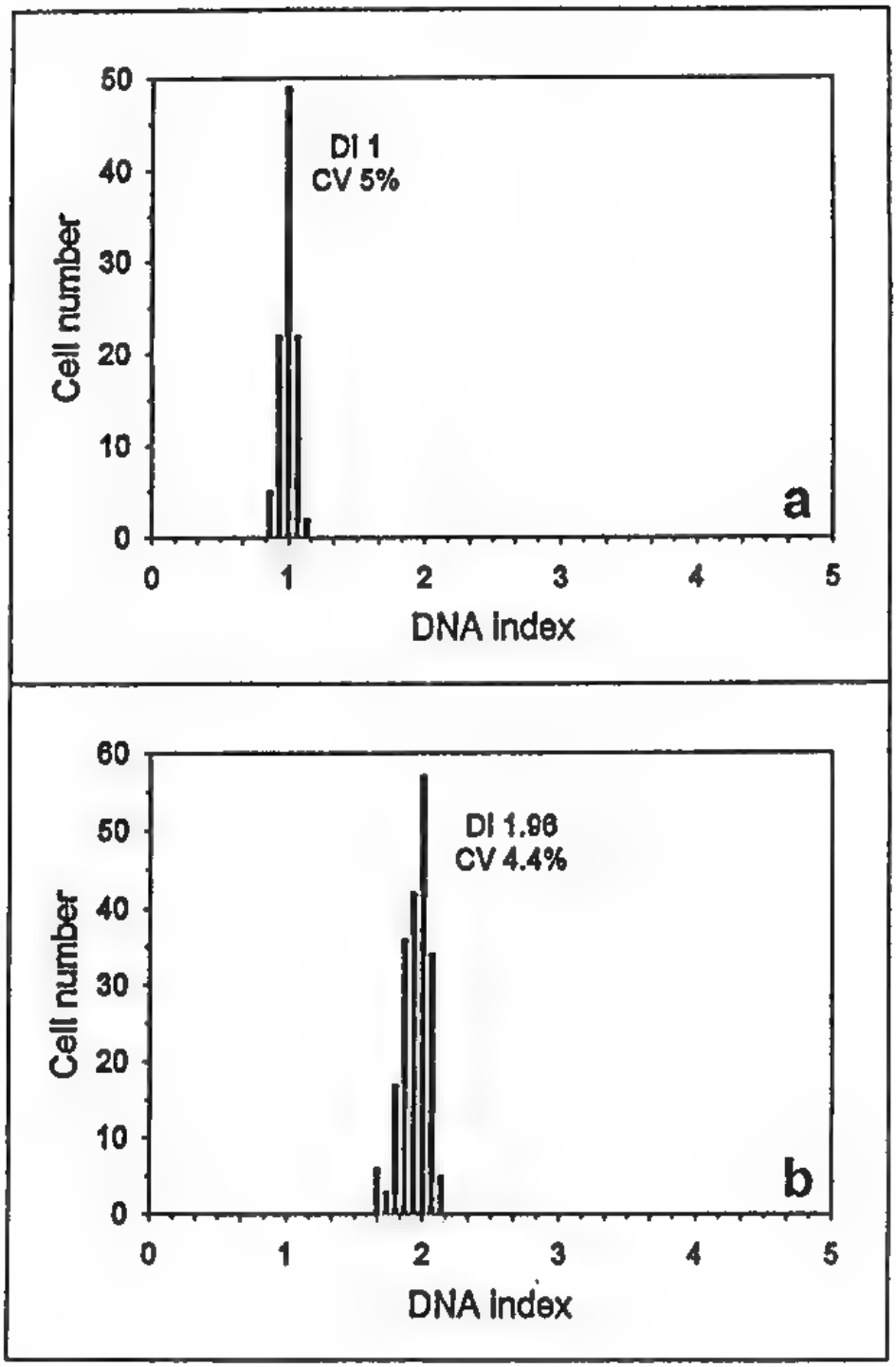

Fig 9 – (a) Frequency distribution of DNA amounts of 100 erythrocyte nuclei of a diploid green toad individual (DNA index DI 1) from population NE Iran. CV, coefficient of variation (%). (b) Frequency distribution of DNA amounts of 200 erythrocyte nuclei of a tetraploid green toad (DNA index DI 1.96) from population Issyk-Kul'. CV, coefficient of variation (%).

Source : MNHN, Paris

animal from the East of the range (Bishkek, Kyrgyzstan), with reference to BACHMANN et al. (1978) and to the DNA measurements (based on flow cytometry) by BORKIN et al. (1986), it can be postulated that the method can be applied also in this region, all the more that tetraploids in this region (Issyk-Kul') are characterised by especially large erythrocytes. The method can help to enlarge the knowledge about distribution of diploid and tetraploid green toads, which is desirable to clarify taxonomic problems (BORKIN et al., 1986).

However, this study has shown that there are populations (Bol'shoi Balkhan) whose values of erythrocyte areas are between most of the other diploid and tetraploid populations. In such cases, or if there are specimens with average values between about 285 and 320 μm^2, it is necessary to use methods such as karyological, cytophotometrical or microdensitometrical analyses (see below).

There were only few records of triploid hybrids in nature, in Danata (PISANETS, 1978; MEŽŽERIN & PISANETS, 1995*a-b*) and near Bishkek (KUZMIN 1995: 187, without reference to the method of ploidy determination), and only few references to "mixed populations", in Dushanbe (ROTH & RÁB, 1986) and in East Kazakhstan (GOLUBEV, 1990, but ploidy determination in this case seems doubtful). Consequently, we first supposed that there were only toads of one level of ploidy at each investigated locality. In none of the populations an inconsistency was found between ploidy of adults and ploidy of larvae using karyological or microdensitometrical methods (see tab. 2; STÖCK, 1995) Among adult animals, a few had average values of erythrocyte areas that were situated in the overlapping range between diploid and tetraploid toads values (marked * in tab. 2). However, all these individuals had the same ploidy as other adult toads and tadpoles of the population. So, it seems very improbable that there were any "mixed population" among those studied.

Variability of erythrocyte areas

In the present study, the number of red blood cells investigated per individual (30 or 50) seems to be large enough, in particular in comparison with literature (OLMO & MORESCALCHI, 1975: 15 cells per individual; GÜNTHER, 1977: 50 cells; KURAMOTO, 1981: more than 20 cells; AUSTIN & BOGART, 1982: more than 20 cells; POLLS PELAZ & GRAF, 1988: 10 cells; SCHRÖER, 1996: 15 cells) The standard deviations within the samples are of the same magnitude as those presented by KURAMOTO (1981) or POLLS PELAZ & GRAF (1988) for some other anuran species.

High variability within the cell sample from a specimen could be caused by the occurrence of immature erythrocytes (erythroblasts) in the peripheral blood of Amphibia (ZAPATA et al., 1982; WELSCH & STARCK, 1986), a presence that has been shown by KHALIL & ELFEKY (1986) to occur in green toads of the *Bufo viridis* complex. Such cells have a tendency to exhibit a circular shape. Among the cells randomly chosen for measurement, this cell type was included too. Apparently the spleen is the principal erythropoietic organ in temperate adult anurans (DUELLMAN & TRUEB, 1994: 180), but due to the activity of the bone marrow as an erythropoietic site during spring (DUELLMAN & TRUEB, 1994: 180), there could be seasonal shifts in the frequency of different erythrocyte sizes. TOKTOSUNOV (1984) noted an increased number of erythrocytes during the cool seasons (autumn, winter, spring) in diploid green toads from Chu valley near Bishkek (Kyrgyzstan). Because of the uniform period of investigations (breeding time) in our study, seasonal effects are thought to be quite constant (see GÜNTHER, 1977). Actually, about half of the individuals from the population Issyk-Kul' were

Source : MNHN, Paris

investigated in autumn, but the range of the values did not diverge from the values in animals investigated in spring. Exclusively some maxima were only detected in spring.

Although the variation range of the erythrocyte areas in all polyploid individuals in the present study is larger than in all diploids (a result similar to GÜNTHER's, 1977, findings in triploid *Rana* kl. *esculenta*, but in contradiction with those of POLLS PELAZ & GRAF, 1988, in the same klepton), a relationship between this phenomenon and polyploidy cannot be clarified. In triploid representatives of the *Ambystoma jeffersonianum* complex, AUSTIN & BOGART (1982) found about the same variability of the erythrocyte areas as in diploid

The erythrocyte measurements by HEMMER et al. (1978) in toads of unknown ploidy from the environs of Dushanbe (Tadzhikistan) could not be compared with the data presented here. On the one hand, these authors measured length and breadth of the ellipsoid and then calculated the ellipse area, and on the other hand they made a Pappenheim staining which might result in a shrivelling of the cells. That might be the reason why their values are smaller than those presented here. Interestingly however, HEMMER et al. (1978) registered high variability in the whole studied sample of toads (i.e. in diploid and tetraploid individuals), a result in agreement with ours. The differences between populations are striking too in toads of the same level of ploidy. In Israel and Greece (areas quite small compared to the giant region taken into consideration here), an enormously high genetic variability was found in diploid *Bufo viridis* (DESSAUER et al., 1975: "the highest yet reported for any vertebrate"; KARAKOUSIS & KYRIAKOPOULOU-SKLAVOUNOU, 1995). Therefore it is not surprising to find a great phenotypic variety in tetraploid green toads, particularly because during evolution, polyploidy was presumably associated with long periods of isolation between many different populations in high mountains (see ROTH, 1986). The phenomenon seems to be confirmed by measurements of DNA contents in erythrocytes (BORKIN et al., 1986): these authors suggested the probable existence of three groups of diploid and two groups of tetraploid toads in Middle Asia. Considering the positive correlation mentioned above, a direct relationship between DNA amount and cell volume (and erythrocyte area) is probable.

Other polyploid amphibians and their differences in erythrocyte areas

Contrary to the situation in the *Ambystoma jeffersonianum* complex, in which diploid and triploid individuals can be distinguished without errors using the very large erythrocytes of the Urodela (AUSTIN & BOGART, 1982), GÜNTHER (1990: 172) and SCHRÖER (1996) mentioned that in some populations of water frogs it is problematical to distinguish between diploid and triploid *Rana* kl. *esculenta* as reported by GÜNTHER (1977). Artificially produced triploid individuals of *Xenopus laevis* exhibited a significant difference between the average values but an overlapping of the absolute values of the largest cell axis compared with diploid forms (GEORGE & LENNARTZ, 1980). MAHONY & ROBINSON (1980) found clear differences in the average values of the erythrocyte areas in the tetraploid Australian leptodactylids *Neobatrachus sudelli* and *N. sutor* compared with those of diploid *N. pictus*; however, this work contains no information on variation range or intrapopular variability. BOGART & WASSERMAN (1972), using two microphotographs, demonstrated the different sizes of erythrocytes in the diploid/tetraploid species pair *Hyla chrysoscelis*/*H. versicolor*, which was confirmed by RALIN (1977) and CASH & BOGART (1978); an extensive study of erythrocyte size in this complex has still not been published.

Source : MNHN, Paris

Relation to age, body size and altitude

The slight increasing of erythrocyte size during ontogenesis till adulthood, as it seems to develop in (diploid and) tetraploid green toads, corresponds to the findings in diploid and triploid *Rana* kl *esculenta* (POLLS PELAZ & GRAF, 1988) whose erythrocyte areas enlarge with increasing body size after metamorphosis. In *Bufo melanostictus*, CHURCH (1961; cited by GÜNTHER, 1977) and BANERJEE (1983, 1988) observed an enlargement of erythrocyte areas with increased body size. In *Bufo spinulosus*, RUIZ et al. (1989) noticed a reduction of erythrocyte area and body size with increasing altitude, whereas erythrocyte number per volume unit was increased (the authors compared individuals from 200 to 2700 m with individuals living above 3200 m) The authors considered this phenomenon an adaptation to the reduced oxygen content in the air of the high mountains. Our investigations do not show any relationship between erythrocyte size and altitude of the habitat. The investigated green toad population living at the highest altitude (Issyk-Kul', 1670 m) displays the highest average value of erythrocyte area. In tetraploid green toads from this area, TOKTOSUNOV (1984) found a higher haemoglobin content (147 g/l) than in diploid lowland populations (112 g/l) and considered this an adaptation of the tetraploids to their mountainous life. It is still unknown whether tetraploid green toads which inhabit high mountains regions above 3500 m (MAZIK et al., 1976; ROTH, 1986; KUZMIN et al., 1988; TOJMASTOV, 1989), and which exhibit in higher altitudes a reduced body size (TOJMASTOV, 1989), have a reduced erythrocyte area too.

MICRODENSITOMETRICAL MEASUREMENTS

DNA amounts

The DNA amount in cells of the tetraploid green toads tested is about twice the content in nuclei of diploid toads. This result is in agreement with those of BORKIN et al. (1986), who found in tetraploids an amount slightly lower than the double of diploids.

Method

This method seems to be the only procedure usable without killing or considerably damaging the animals to determine unequivocally their ploidy. The Feulgen process cited above (HORNER & MACGREGOR, 1983) is suitable for erythrocytes in fresh blood smears from Middle Asian green toads. Up to now it is unknown how long blood smears could be stored if they were fixed as mentioned before staining them with the Feulgen reaction. A test is in preparation. If it turns possible to store blood smears some time without loss in quantitative Feulgen staining, this method could become a key to collect data about distribution of different ploidy types in Middle Asian green toads without killing the specimens. Such a method would be most useful in faunistics and other field investigations.

Source : MNHN, Paris

RÉSUMÉ

Les tailles des érythrocytes de populations de crapauds verts (complexe de *Bufo viridis*) diploïdes et tétraploïdes de l'Asie centrale (Iran, Turkménistan, Ouzbékistan et Kirghizstan) sont comparées en mesurant leur surfaces de projection à partir de frottis sanguins. Les valeurs moyennes de ces surfaces sont significativement différentes entre les deux types de crapauds. Dans quelques populations les valeurs moyennes sont intermédiaires entre celles des animaux diploïdes et tetraploïdes. La variabilité des surfaces des érythrocytes par rapport à l'âge, la taille et l'altitude, est discutée à la lumière de la littérature sur les crapauds verts et les autres anoures polyploïdes. La coloration des noyaux des érythrocytes par la réaction de Feulgen et la détermination microdensitométrique du contenu de l'ADN sont décrites. Cette méthode est préconisée pour obtenir des résultats clairs sans sacrifier les animaux.

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What is the ecological significance of laboratory temperature selection in anuran Amphibia?

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Temperature selection by a toad (*Bufo viridis*), was studied in the laboratory. This species covers a wide geographical distribution, and is exposed to different temperatures in its natural habitats. A temperature gradient was constructed within a 1.2 m tunnel in the laboratory, and the toads selected a preferred temperature around 27°C, when allowed free choice. Acclimatization to an ambient temperature of 16°C lowered the selected temperature by some 3-4°C. As reported in the literature, many anuran species with different climatic backgrounds share a relatively narrow range of selected preferred temperature in the laboratory. We suggest that the laboratory selected temperature represents an upper set-point that provides anurans with the information to evade high lethal temperatures.

Introduction

Despite their high vulnerability to ambient conditions, amphibians have invaded many regions of the globe in specific niches. Because water permeability of their moist skin is high, it demands that they avoid permanently hot and dry regions (Jørgensen, 1950). Physiological regulatory temperature control in amphibians is poor or absent (Brattstrom, 1979), but they use behavioural means to select optimal temperature (Heath, 1970). Some amphibians that were studied in the field regulate a relatively constant temperature behaviourally (Lillywhite, 1970; Sinsch, 1984), while some species that inhabit dry regions burrow (Katz, 1989, Pinder et al., 1992). In other species, protective mechanisms were developed, such as waxing or cocooning (Loveridge, 1976), that greatly reduce the water permeability of their integument (Shoemaker & Nagy, 1977) In either case, in hazardous environments, most amphibians seek shelter and only a few, adaptable species, face the ambient conditions and remain active (Shoemaker & Nagy, 1977; Katz, 1989).

Bufo viridis is one of a few adaptable anurans that can be acclimatized to a wide variety of osmotic and thermal environmental conditions (Tercafs & Schoffeniels, 1962; Katz & Gabbay, 1986; Katz & Gil, 1997). It has a wide area of distribution, extending from temperate south Scandinavia to hot and arid regions in Israel, Egypt and north Africa. *Bufo viridis* is nocturnal and is normally active throughout the year; it burrows, but only intermit-

Source : MNHN, Paris

tently, in conditions of water shortage (DEGANI et al., 1984; KATZ & GABBAY, 1986; HOFFMAN & KATZ, 1994). In this study we investigated the temperature selection of *Bufo viridis* in laboratory conditions, and examined the effects of temperature acclimatization and dehydration on this behaviour.

MATERIALS AND METHODS

Bufo viridis from Israel (more than 20 individuals, belonging to both sexes) were used in this study. Average weight of the toads was 34 g (ranging between 25 and 40 g), and they were kept in the laboratory at room temperature (20-24°C) with free access to tap water. The toads were force-fed ox liver weekly, maintaining constant weight.

Temperature selection of the toads was determined in a temperature gradient that was produced in an opaque plastic tunnel (130 × 14 × 14 cm) heated by two blackened 75 W bulbs, one at each end, and cooled from the outside with dry ice at about a third of the length. Thus, an asymmetric temperature gradient was produced, with a profile that spans over nearly 15°C as shown in fig. 1. Relative humidity in chamber did not exceed 60 %, and the tunnel was dry. The toads were placed singly into the centre of the tunnel, and were allowed 30 min to settle. Changes of position were recorded (through a mirror, to avoid external interference) for several hours, and time spent at various distances from one end were calculated in relation to ambient temperature.

The relationship between body and ambient temperatures was studied separately, in a chamber (10 × 6 × 6 cm) that was kept in a temperature-controlled water bath. The ambient temperature was changed stepwise, by 2-5°C each time, only after the body (and skin) temperature was stabilized (which took no less than 30 min). Temperature was measured with a precision of 0.1°C by a thermistor (YSI, Ohio telethermometer) that was inserted about 2 cm into the cloaca.

RESULTS

Figure 2 shows that, in laboratory conditions, body temperature in *Bufo viridis* (4 specimens) was related almost linearly (in the range 5-35°C) to the ambient temperatures at steady state. The slope of the regression line over the whole range was $y = 0.88\ x - 0.07$; $r^2 = 0.95$ (y is body temperature in degrees centigrade and x is ambient temperature). This relationship deviated at the higher temperatures (range 25-35°C), having a steeper regression line ($y = 1.12\ x - 7.73$; $r^2 = 0.78$).

Toads that were acclimatized at room temperature (ca 22°C), selected a preferred ambient temperature of 27-28°C (fig. 3). The toads spent most of the time in the gentler

Source : MNHN, Paris

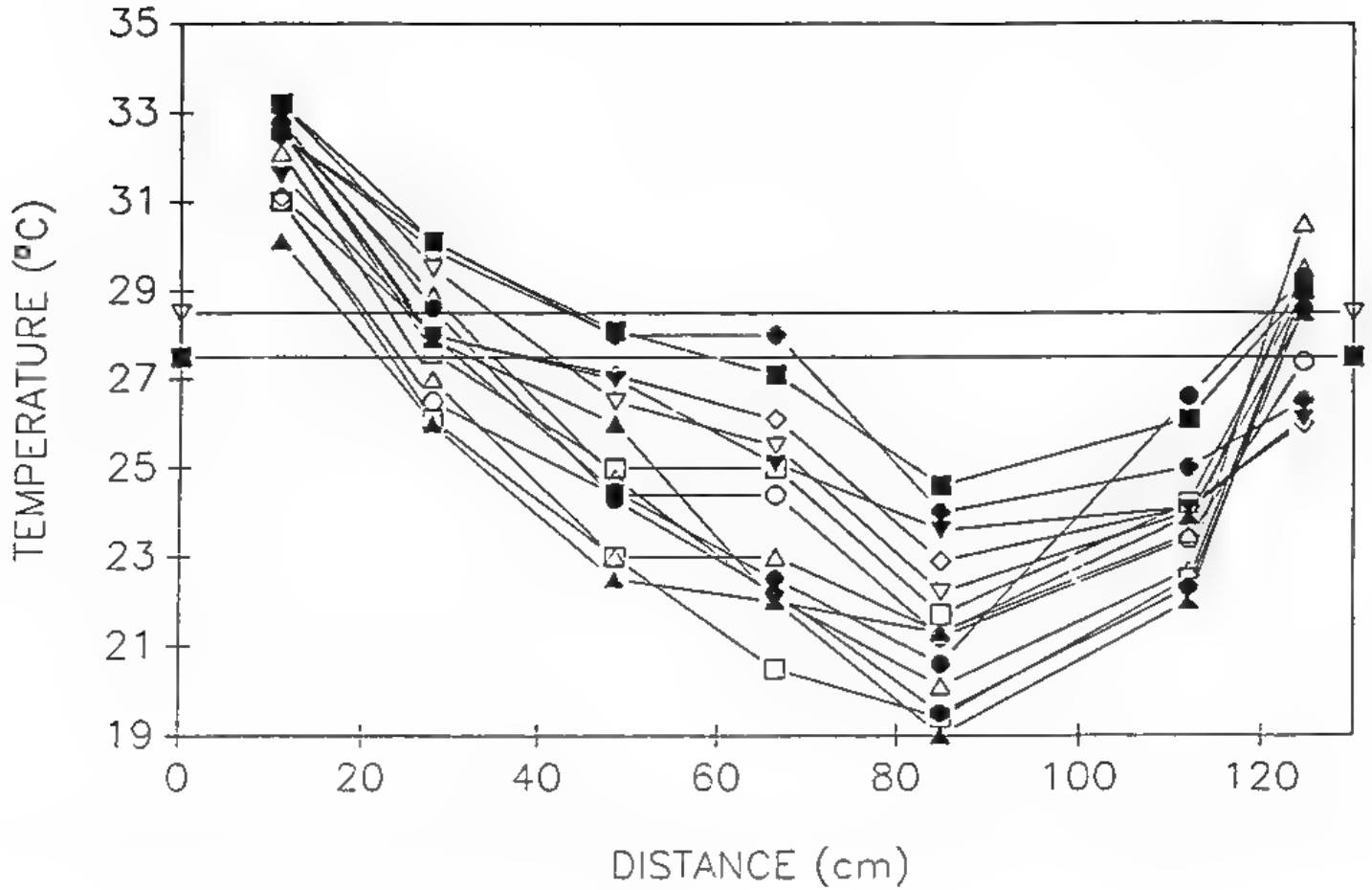

Fig. 1. – Temperature gradients along the (120 cm) tunnel, as recorded in the individual experiments (n = 13). An asymmetric gradient was created by placing two heat sources at each end of the tunnel, and a cooling region located in the third of the distance between them Note that the toads selected voluntarily a narrow temperature range, indicated by the two horizontal lines.

Source : MNHN, Paris

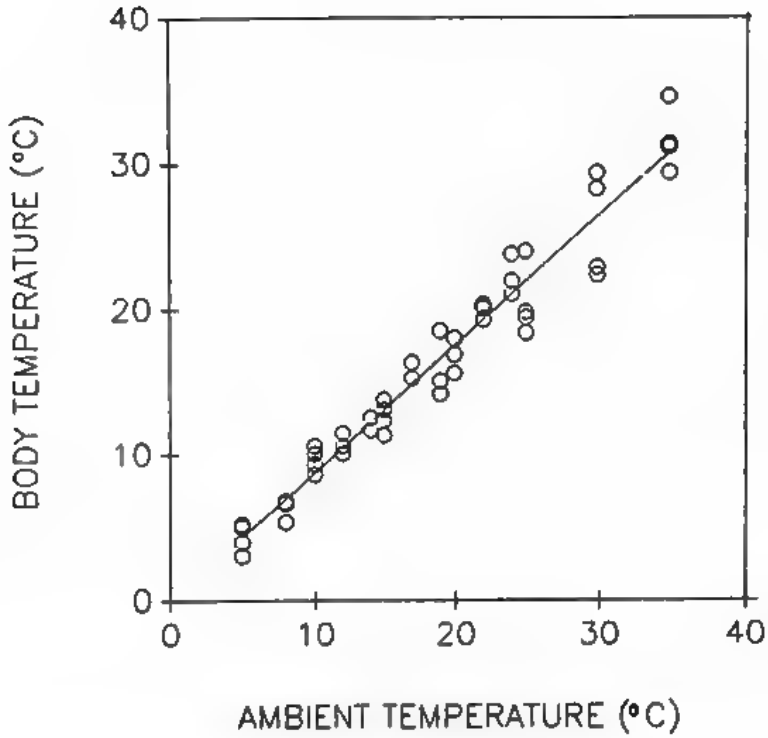

Fig. 2. Relationship between body and ambient temperature in *Bufo viridis*. The regression line equation is y = 0 88 x -0 07, r^2 = 0 95 (5 toads acclimatized at room temperature, ca. 22°C). Weight of the animals was 30-35g.

gradient, with about 20 % of the total time at temperatures below 27°C. As shown in fig. 1, the selected temperature (27-28°C) occupied no more than 15 % of the total temperature profile, including part of the steeper gradient in the tunnel. In control experiments with no temperature gradient, 3 toads that were placed individually in the tunnel explored it over its length and stationed themselves at the ends for most of the time. No other preferred points were observed. This preference disappeared in the presence of the thermal gradient.

Acclimatization of the toads at 16°C for over 3 weeks resulted in a shift in the distribution pattern of the selected temperature (fig. 3), so that now 75 % of total time was spent below 27°C. The difference in the preferred temperature between the two conditions was highly significant ($P > 0.001$; $n = 6$). Fast dehydration (by approximately 15 % gross weight in less than a day) also changed the distribution pattern of preferred temperature, with shorter time spent above 27°C (fig. 4).

Source : MNHN, Paris

DISCUSSION

Our experiments are in accord with previous observations on other species (HUTCHISON & DUPRE, 1992, for a review) and show that when the ectothermic terrestrial anuran *Bufo viridis* is allowed a free choice of temperature in the laboratory, it selects a relatively narrow range of ambient temperatures and spends most of the time at 26-28°C. Preliminary unpublished observations that we made on the African species *Bufo regularis* gave similar results.

Body temperature of amphibians is determined largely by external physical conditions, including ambient temperature, relative humidity (MELLANBY, 1942; MALVIN & WOOD, 1991) and resistance to water loss (SNYDER & HAMMERSON, 1993). Their body temperature is usually somewhat lower than ambient, due to continuous evaporation, although in a number of montane species body temperature is maintained nearly always above ambient (LILLYWHITE, 1970; VALDIVIESO & TAMSITT, 1974; SINSCH, 1989). In natural conditions, amphibians employ mostly behavioural means to regulate their body temperature (LILLYWHITE, 1970); they either bask, as e.g. in *Hyla labialis* (VALDIVIESO & TAMSITT, 1974), or they shelter, to avoid over-heating. They must optimize between conflicting demands of temperature-dependent biochemical reactions and water balance (BRATTSTROM, 1979).

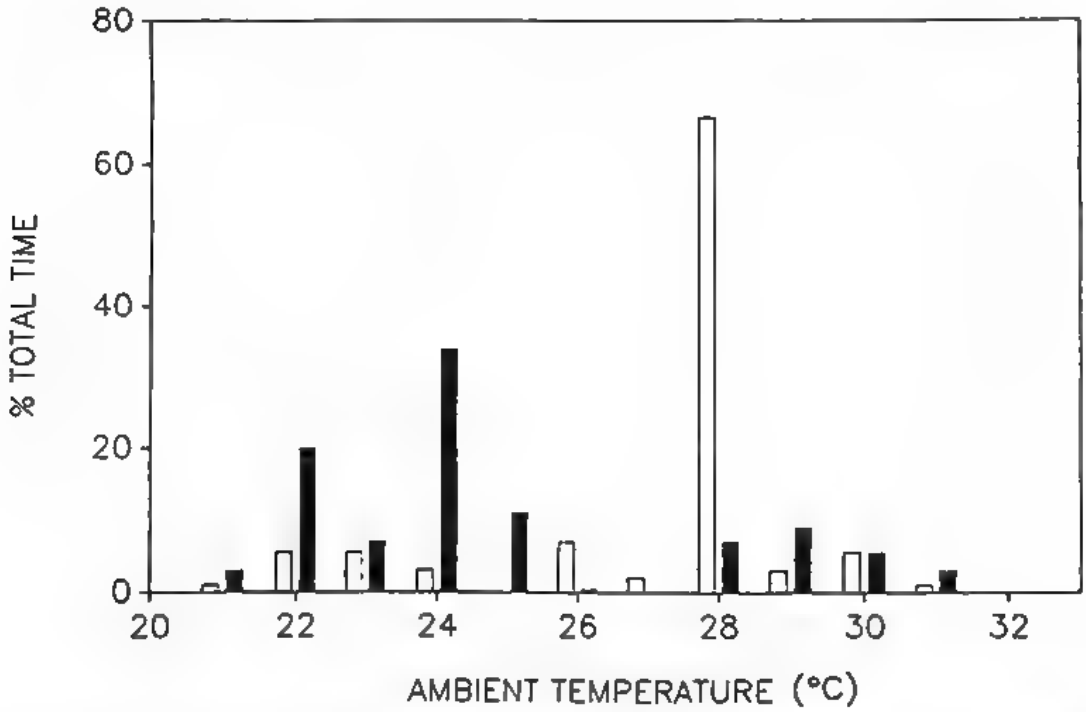

Fig. 3. – Effect of temperature acclimatization on the preferred ambient temperature in *Bufo viridis*. Summed results of 6 sessions. Open bars, animals acclimatized at room temperature (ca. 22°C); dark bars, animals acclimatized at 16°C.

Source : MNHN, Paris

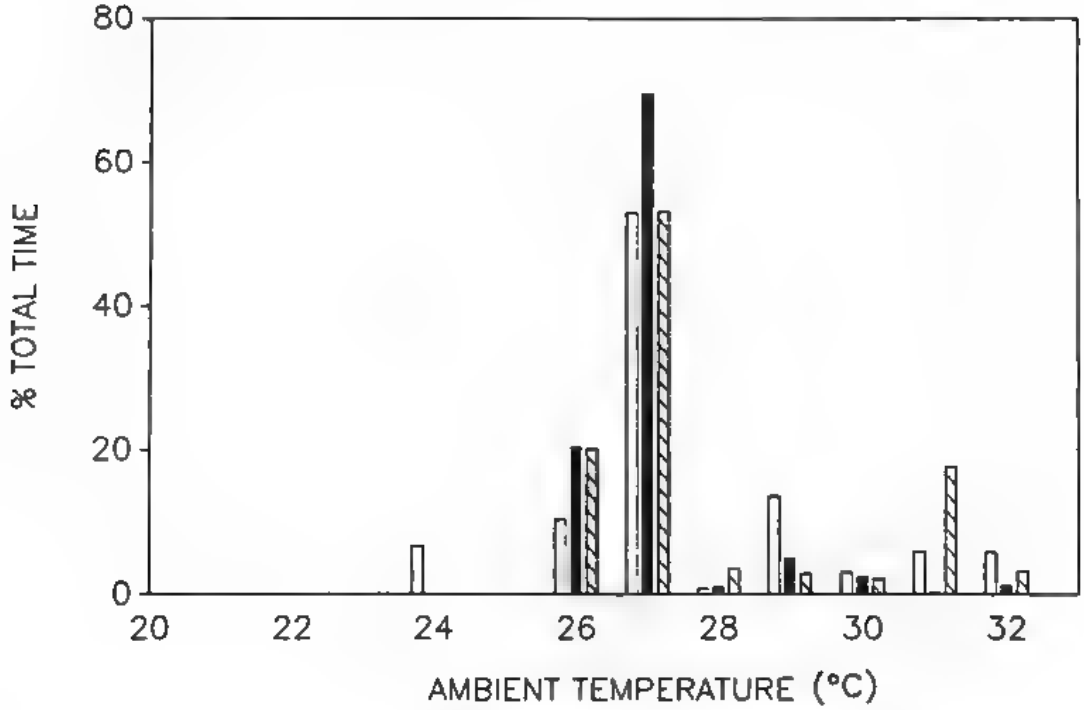

Fig. 4. Effect of dehydration on preferred ambient temperature in *Bufo viridis* Summed results of 4 individuals on 4 separate occasions in each condition. Open bars, acclimatized at room temperature (ca. 22°C), dark bars, dehydrated by 15 % for 16 h at room temperature, hatched bars, dehydrated, water bath present in the terrarium

Selected preferred temperature seems to be an inherent property, common to many or most anurans, and does not seem to be related to the species ecological background. *Bufo regularis*, for example, is an African species that cannot survive at temperatures below 8°C (KATZ & GIL, 1997), and yet it displayed a similar preferred temperature as did *Bufo viridis*, which survives at near freezing temperature. In many species, the laboratory preferred temperature and the temperature activity in their habitat were found to be quite removed from each other (CLAUSEN, 1973), although in some species these temperatures met with one another, as was found in the aquatic *Rana catesbeiana* (LILLYWHITE, 1970, 1971). The many studies of laboratory preferred temperature, performed on a large number of amphibians, revealed a narrow range of preferred temperature (between 26 and 30°C) which was selected by more than 75 % of adults among over 40 species of anurans that were tested (HUTCHISON & DUPRE, 1992). Only a few species, including juveniles, chose within the range 16-25°C, while ca. 10 % opted for 31-34°C; this was so, despite the large differences in the experimental methods and acclimatization protocols. This is even more pronounced in the data summarized by STRUBING (1954), where only two anurans (*Bombina* spp.) deviated from the 26-33°C range of 34 listed species. Therefore, temperature selection in the laboratory does not reflect the thermal conditions of natural habitats of the animals which vary widely. Rather, it indicates an essential requirement of some fundamental importance to anurans. Some investigators suggested that the amphibian preferred temperature could represent ectother-

Source : MNHN, Paris

mic equivalence of the endothermic set-point (HUTCHISON & DUPRE, 1992), but this thesis does not seem to be tenable.

If the selected preferred temperature is not related to the thermal background of the animal in nature, what then could it signify? We propose that the laboratory-selected temperature represents a set-point that demarcates a limit to the tolerated temperature. This is supported by the finding that the upper lethal temperature of most toads and frogs – with the notable exceptions of *Phyllomedusa sauvagei* (~40°C) and *Bufo calamita* (~37°C) – is 32-33°C (KIRK & HOGBEN, 1946), much lower than in reptiles, which are also ectothermic. Although amphibians do not possess physiological thermoregulatory mechanisms, they seem to be equipped with a temperature set-point and with cold- and warm-sensitive neurons in the skin (CABANAC & JEDDI, 1971; DUPRE et al., 1986). These are necessary components in any feed-back control system, and provide information that can be used either in physiological temperature regulation or, if not available, for activation of behavioural escape mechanisms. Lacking effective physiological thermoregulatory mechanisms, physical activity and metabolic rate in Amphibia will continue to increase as ambient and body temperatures increase. Therefore, an upper temperature set-point is required to supply safety command to evade lethal temperatures.

In conclusion, we found that *Bufo viridis* and *Bufo regularis* selected a similar preferred temperature in the laboratory, although they show entirely different sensitivity to temperature. Published data and the present study indicate that, in the laboratory, many or most anurans select a preferred temperature from within a relatively narrow range. This temperature is often far removed from, and higher than, the one encountered in the field during their periods of greatest activity. It is suggested that the preferred temperature represents an upper set-point that provides the animals with the information to evade high temperatures.

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Un cas de néoténie, dans un bâtiment désaffecté, chez le triton alpestre, *Triturus alpestris apuanus* (Salamandridae)

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A population of *Triturus alpestris apuanus* of the Apuane Alps (Tuscany, Italy) was studied during July 1994. The particularity of this population is that it breeds in an artificial site, in the dark and under cover, in a deserted building. Moreover some neotenic newts were discovered. The recent colonisation and the instability of this biotope indicate a form of labile neoteny, appearing and disappearing spontaneously.

INTRODUCTION

La néoténie (KOLLMANN, 1884) est la conservation des caractéristiques de l'état larvaire à un stade de développement plus avancé que la normale La néoténie totale ou pédogenèse concerne des animaux devenus matures dans cet état, tandis que la néoténie partielle ne s'applique qu'à un retard de la métamorphose.

La néoténie est un problème qui soulève de nombreuses questions. On peut en effet se demander, d'une part, quelle est la cause de l'apparition d'individus néoténiques, et d'autre part, comment ces derniers peuvent se maintenir en syntopie avec des individus métamorphosés (et inversement). Quelques réponses ont été données à ces questions en ce qui concerne le genre *Triturus*. Suite à l'observation de populations néoténiques dans des lacs à régime stable en altitude, certains auteurs ont attribué le maintien du pédomorphisme (néoténie au sens large, impliquant un retard du développement de caractères somatiques et/ou sexuels: DUBOIS, 1985) à une prétendue hostilité du milieu terrestre (WILBUR & COLLINS, 1973; DUELLMAN & TRUEB, 1985). SVOB (1965) a montré qu'une faible luminosité associée à une basse température pouvait entraver la métamorphose de *Triturus alpestris montenegrinus*, tandis que l'obscurité seule provoquait la mort des tritons (à une température de 18-20°C). Il suggère ainsi que la température basse est une des conditions pour la résistance des tritons néoténiques à l'action inhibitrice d'une luminosité insuffisante. Les lacs d'altitude, pour la plupart froids et obscurs, offrent ainsi des conditions favorables à l'apparition de la néoténie. Mais le problème serait trop simple s'il

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s'arrêtait là. En effet, il existe des lacs d'altitude entourés d'un milieu prétendu hostile où l'on ne rencontre pas de tritons pédogénétiques (BREUIL, 1986). Selon BREUIL (1992), celui-ci ne le serait pas obligatoirement, et ce notamment en milieu karstique où les très nombreuses cavités offriraient des refuges adéquats aux animaux. D'un autre côté, on a récemment découvert des populations néoténiques dans des milieux instables voire temporaires et entourés d'un milieu terrestre apparemment favorable. Il en est ainsi en ce qui concerne les tritons alpestres néoténiques du Nevesinjsko Polje en Bosnie (DZUKIC & KALEZIC, 1984) et du Lago dei Due Uomini en Calabre, ce dernier lac s'asséchant même en été (DUBOIS & BREUIL, 1983; DUBOIS, 1983; BREUIL, 1986). L'assèchement des points d'eau contenant des populations néoténiques est un problème difficilement soluble. Certains auteurs parlent dans ce cas de néoténie labile, celle-ci pouvant apparaître et disparaître spontanément (FUHN, 1963; GABRION et al , 1977, 1978; BREUIL, 1992).

Dans la présente note, nous rapportons l'observation d'une petite population néoténique dans un milieu instable et obscur.

MATÉRIELS ET MÉTHODES

La population observée occupe un complexe de bacs en béton et de canaux partiellement remplis d'eau dans un bâtiment en construction désaffecté depuis environ 5 ans (fig. 1). Ce site devait devenir une station thermale, mais les travaux ont dû être arrêtés à la suite d'impératifs budgétaires. Il est situé près de Castelnuovo di Garfagnana en Toscane (Italie) et plus précisément à quelques mètres de l'étang "Il Bagno" (Lago di Prà di Lama) (UTM: 32TPP1387). L'altitude de la station est de 357 mètres. D'après FERRACIN et al. (1980) ainsi que BREUIL (1986), cet étang contient une population de *Triturus alpestris apuanus*, mais ces auteurs n'y ont trouvé aucun individu néoténique. Toutefois, nous n'excluons pas qu'il puisse exister des animaux néoténiques dans cette station. En effet, le pédomorphisme n'est pas un phénomène exceptionnel chez *T. a. apuanus*: des cas en ont été relevés dans les provinces de Torino, Cuneo, Savona, Genova, Piacenza, Arezzo et Rieti (ANDREONE & DORE, 1991) et de Lucca (FERRACIN et al., 1980).

Lors de ma visite, le 17 juillet 1994, 17 bacs ainsi que le système "d'égouttage" contournant le bâtiment contenaient de l'eau. Une vingtaine d'autres bacs et une partie du canal d'égouttage étaient à sec. La profondeur des bacs et du canal est de 20 cm, mais celle de l'eau n'est que de 3 à 9 cm. La superficie des bacs est de 13 à 27 m^2 selon ceux-ci. Il existe des communications (tuyaux) entre certains des bacs et le canal d'égouttage, mais le niveau d'eau ne les atteint pas toujours. Néanmoins, ce dernier a probablement déjà été plus élevé, permettant ainsi des échanges entre les bacs. La lumière naturelle pénètre à l'intérieur d'une partie du bâtiment, mais le canal d'égouttage est quant à lui presque totalement obscur.

La majorité des tritons alpestres, aussi bien les adultes que les juvéniles et les larves, ont été capturés bac après bac et relâchés directement. Tous les individus capturés ont été mesurés vivants, sans avoir été anesthésiés, du museau à l'extrémité de la queue (L). Il semble que la marge d'erreur n'excède pas le millimètre.

Nous avons distingué cinq groupes au sein de la population observée: (1) les larves (individus présentant des branchies bien développées, mesurant moins de 65 mm et ne possédant aucune tache gulaire); (2) les juvéniles (tritons métamorphosés mais encore immatures); (3) les subjuvéniles (tritons immatures en fin de métamorphose – larves ou

Source : MNHN, Paris

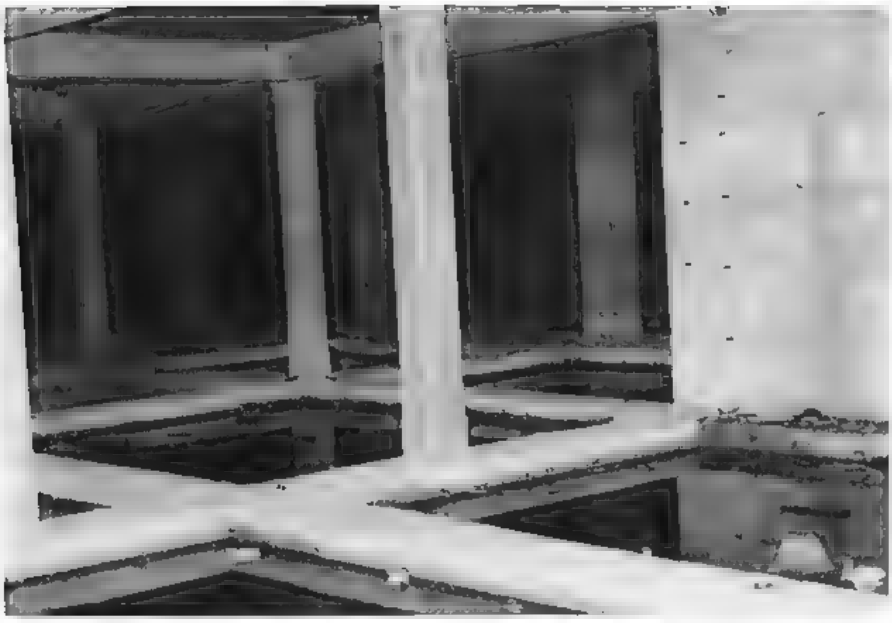

Fig. 1. – Vue de l'intérieur du bâtiment montrant les bacs où ont été trouvés les spécimens néoténiques de *Triturus alpestris apuanus*.

néoténiques – présentant la coloration et l'habitus des juvéniles, mais ayant encore des branchies); (4) les néoténiques (tritons présentant un phénotype larvaire mais de grande taille – plus de 60 mm – et possédant des taches gulaires); (5) les adultes métamorphosés (tritons métamorphosés, de grande taille – plus de 70 mm pour les mâles, plus de 75 mm pour les femelles – , et supposés matures selon l'examen des caractères extérieurs).

RÉSULTATS ET DISCUSSION

Sur les 131 individus capturés dans l'eau, on dénombre 99 larves, 6 subjuvéniles, 13 juvéniles, 6 néoténiques, 2 adultes mâles et 5 adultes femelles (tab. 1).

La répartition spatiale des différents membres de la population est assez hétérogène: certains bacs ne contiennent que des larves alors que d'autres contiennent également des juvéniles, des adultes et des néoténiques. On constate ainsi que presque la moitié des adultes se trouvent rassemblés dans le même bac, faiblement éclairé, d'une superficie de 28 mètres carré et d'une profondeur maximale de 4 cm.

Les larves sont en moyenne de grande taille, près des deux tiers mesurant plus de 50 mm (tab. 1). Certaines de ces larves sont d'ailleurs à un stade assez proche de celui des néoténiques. Les 6 néoténiques (tout comme les métamorphosés) ont la partie gulaire tachetée, légèrement chez le plus petit d'entre eux (L = 60 mm) (fig. 2).

Source : MNHN, Paris

Tab. 1. - Valeur moyenne, écart-type et valeurs extrêmes (en millimètres) de la longueur totale dans les 6 groupes de la population de *Triturus alpestris apuanus* étudiée.

Stade (n)	Moyenne	Ecart-type	Valeurs extrêmes (minimum-maximum)
Larves (99)	50,8	6,3	30-63
Subjuvéniles (6)	51,8	16,3	45-85
Juvéniles (13)	70,7	10,1	49-80
Néoténiques (6)	75,8	11,1	60-88
Femelles adultes (5)	99,0	11,7	71-107
Mâles adultes (2)	74,5	-	71-78

Fig 2. — Spécimen néoténique de *Triturus alpestris apuanus* d'une longueur totale de 80 mm

La population observée occupe un habitat aux conditions très particulières. En effet, il s'agit d'un milieu obscur et frais comme c'est le cas des lacs d'altitude où l'on rencontre parfois des populations néoténiques. Ces paramètres physiques, joints à la faible densité de proies, ont très certainement ralenti la croissance des animaux et peut-être ainsi entravé la métamorphose. Cette supposition est appuyée par les expériences de Svob (1965) qui ont montré qu'une telle situation pouvait effectivement entraver la métamorphose des tritons. In natura, dans les milieux d'altitude stables, on constate également que les larves ont tendance à passer l'hiver dans l'eau (Breuil, 1992). Toutefois le milieu étudié diverge de ces milieux d'altitude entre autres par son instabilité et son faible volume d'eau. De plus, il est d'origine extrêmement récente (moins de 5 ans). Ces faits nous amènent à penser qu'il

Source : MNHN, Paris

s'agirait d'une néoténie de type labile, pouvant apparaître et disparaître spontanément. Celle-ci aurait été induite par les conditions particulières qu'offre ce milieu artificiel, et qui correspondent un peu à celles rencontrées dans certaines grottes. BREUIL & PARENT (1987) ont d'ailleurs observé des *Triturus alpestris veluchiensis* dans une nappe d'eau souterraine. Les cas de néoténie chez *T. a. apuanus* étant relativement fréquents, il est probable qu'ils aient une certaine base génétique sous-jacente, celle-ci ne s'exprimant que lors de conditions environnementales particulières.

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Contents

Carlos Alberto Gonçalves DA CRUZ, Ulisses CARAMASCHI & Eugênio IZECKSOHN
The genus *Chiasmocleis* Méhely, 1904 (Anura, Microhylidae) in the Atlantic Rain Forest of Brazil, with description of three new species ... 49-71

Matthias STÖCK & Wolf-Rüdiger GROßE
Erythrocyte size and ploidy determination in green toads (*Bufo viridis* complex) from Middle Asia 72-90

U. KATZ, Joy HOFFMAN & Nira GIL
What is the ecological significance of laboratory temperature selection in anuran Amphibia? 91-98

Mathieu DENOËL
Un cas de néoténie, dans un bâtiment désaffecté, chez le triton alpestre, *Triturus alpestris apuanus* (Salamandridae) 99-103

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